MICROSPORIDIAN PARASITES OF MOSQUITOES; ULTRASTRUCTURE, LIFE CYCLES AND RELATIONSHIPS

By

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To E. I. Hazard

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Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

MICROSPORIDIAN PARASITES OF MOSQUITOES; ULTRASTRUCTURE, LIFE CYCLES AND RELATIONSHIPS

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Two concurrent sporulation sequences of the microsporidium Culicosporella lunata occur in larvae of the mosquito Culex pilosus. One sequence involves meiosis and produces meiospores while in the other sporogonial plasmodia form diplokaryotic spores. The ultrastructural characterization of the developmental sequences and spore morphology have clearly demonstrated an affinity to other members of the Amblyosporidae. This species differs, however, in that the meiotic sequence is abortive and the spores responsible for horizontal transmission to the mosquito are diplokaryotic. A previously unknown sporulation sequence in female adults is revealed that produces binucleate spores. This strongly implicates the

involvement of transovarial transmission in the developmental cycle of this species.

A new Amblyospora sp. from the predacious Australian mosquito Culex halifaxi is described based on both light and electron microscopy. Two concurrent sporulation sequences in larvae result in the production of morphologically distinct spores. Meiospores and uninucleate spores can presumably function in horizontal transmission to the mosquito host by different pathways. A meiotic sequence produces meiospores that may infect a copepod intermediate host to form spores responsible for horizontal transmission to larval mosquitoes. Concurrently, uninucleate spores from dissociation are formed in larvae and may directly infect the mosquito host. Speculation on the implications and flexibility that this unique developmental cycle affords the parasite is discussed.

For Amblyospora californica in Culex tarsalis, evidence at both the light and ultrastructural levels documents the previously unknown horizontal sequence of development in the mosquito host. Meiospores of A. californica are infectious to an intermediate copepod host Macrocyclops albidus. The resulting spores are infectious to larval C. tarsalis giving rise to a sequence that involves gametogenesis, plasmogamy and nuclear association to form binucleate spores in female adults.

Using Lacto-Aceto-Orcein squashes, the chromosome number for an Amblyospora sp. in Culex salinarius is shown to be nine, the same as A. californica. Additionally, Amblyospora sp. from C. salinarius was successfully transmitted to C. tarsalis and completed its full developmental cycle. These findings indicate that this Amblyospora sp. and A. californica are closely related with the strong probability that they represent only one species.

CHAPTER I
MICROSPORIDIAN PARASITES OF MOSQUITOES

Hesse (1904) was probably the first researcher to document a true microsporidian parasite of a mosquito when he described Thelohania legeri from Anopheles maculipennis Meigen. Perhaps the most comprehensive study of these mosquito parasites at the time was a series of eight publications by Kudo (1921, 1922, 1924b, 1924c, 1925a, 1925b, 1929, 1930) under the general title "Microsporidia Parasitic in Mosquitoes." This series was notable in that extensive details and drawings on the developmental stages of various microsporidia provided much new information which has proven to be remarkably accurate. But given the minute size and complexity of these parasites, along with the limitations of the instruments at that time, many details were understandably misinterpreted or overlooked. For these reasons, many ideas and conclusions about the development and relationships of microsporidian parasites of mosquitoes were accepted and perpetuated by subsequent researchers. Accurate information was slow to develop for numerous reasons, but a primary hindrance was the inability to transmit these parasites to the mosquito host. It was not until this obstacle was overcome that significant

advancements were made in understanding the complex life cycles of these mosquito parasites.

The first revelation was the discovery of dimorphism and the role of transovarial transmission in some species of Thelohania found parasitizing Anopheles mosquitoes, an important finding for the taxonomy of the Microsporidia (Sprague, 1976). Hazard and Weiser (1968) reported that a binucleate spore in the adult female was responsible for transmitting the pathogen to progeny. Their studies revealed that in infected male larval progeny, uninucleate spores (meiospores) were produced within a sporophorous vesicle. In infected female progeny, however, spore development was delayed until pupation and adult emergence. In these females, binucleate spores of the original type are produced to repeat the cycle.

The role of the meiospores was an enigma as all attempts to infect the mosquito host were unsuccessful. The means by which the parasite entered new host individuals remained a mystery until the discovery that meiospores formed in larvae were infectious to a copepod intermediate host (Sweeney et al., 1985). When ingested by mosquito larvae, the spores formed in the copepod intermediate host initiated a sequence of development that ended with binucleate spores in the adult female mosquito. The developmental cycles of some of these microsporidian parasites of mosquitoes have therefore been shown to involve both vertical (transovarial)

transmission and horizontal (per os) transmission affecting two generations of the mosquito host with the involvement of an intermediate host.

Recent studies on microsporidian parasites of mosquitoes have provided much new information on life cycles in general as well as specific details of developmental sequences at both the light and ultrastructural levels (Andreadis, 1983a & b, 1985a & b, 1988a & b; Andreadis and Hall, 1979a & b; Becnel, 1986; Becnel et al., 1986, 1987, 1989; Chen, 1988; Hazard and Anthony, 1974; Hazard and Oldacre, 1975; Hazard et al., 1985; Sweeney et al., 1985, 1988). This dissertation generates new information on two established microsporidian species as well as the formal description of a new species from an Australian mosquito. Possession of this new information in addition to the established knowledge of these microsporidian parasites provides the opportunity to discuss the relationships of this group of mosquito parasites.

For Amblyospora californica in Culex tarsalis, I will document the previously unknown horizontal sequence of development in the mosquito host initiated by spores formed in the copepod intermediate host. Evidence at both the light and ultrastructural level confirms previous reports in A. connecticus (Andreadis, 1988a) and A. dyxenoides (Sweeney et al., 1988) as well as establishing new characters for the type species A. californica. The results of cross

transmission tests with an Amblyospora sp. from C. salinarius into C. tarsalis and the implications of these findings will also be discussed.

I will formally describe a new Amblyospora sp. from Culex halifaxi, a predacious Australian mosquito. This unique microsporidium is described based on both light and electron microscopy from a sequence of development observed in the larval stage. This species appears to be an intermediate form with both meiospores and uninucleate spores from dissociation found concurrently in the larval mosquito which, presumably, acquired the infection via transovarial transmission (Becnel, 1986). While certain aspects of the developmental cycle can only be speculated upon until additional developmental cycle information is acquired, formal description is justified due to the uniqueness of the observed sporulation sequence.

An ultrastructural study on the known portion of the life cycle of Culicosporella lunata in Culex pilosus will provide confirmatory evidence for previous studies at the light level (Hazard and Savage, 1970; Hazard et al., 1984) as well as establishing characters needed for comparisons at the ultrastructural level. An abortive meiotic sequence and the concurrent formation of spores morphologically like those formed in the copepod intermediate host (except that they are diplokaryotic) establish yet another variation of microsporidian parasites of mosquitoes.

There have been few studies dealing with the evolution and phylogenies of the microsporidia (Hazard, 1977a; Sprague, 1977; Issi, 1978; Larsson, 1986; Vossbrink and Woese, 1986; Vossbrink et al., 1987) primarily due to both a paucity of information on most groups and lack of consensus as to which characters are most important taxonomically (Larsson, 1988). Some heterosporous microsporidia parasitizing mosquitoes will be compared to determine which characters are most useful for determining relationships. The presence or absence as well as similarities and differences in developmental stages and sequences may provide clues to the developmental direction of species within this group.

CHAPTER II
ULTRASTRUCTURE OF *Culicosporella lunata* IN THE MOSQUITO *Culex pilosus* WITH NEW INFORMATION ON THE LIFE CYCLE

Introduction

Culicosporella lunata (Hazard and Savage, 1970) Weiser, 1977, a microsporidian parasite of the mosquito *Culex pilosus* (Dyar and Knab), has been recognized as unique since its discovery. Based on rosette-shaped sporogonial plasmodia and spore morphology, this microsporidium was placed in the genus *Stempellia* Leger and Hesse by Hazard and Savage, 1970. Later, Weiser (1977) reassigned this species and designated it as the type for a new genus, *Culicosporella*. After a more comprehensive study with the light microscope, Hazard et al. (1984), redescribed *C. lunata* reinterpreting some of their earlier observations. The details of a previously overlooked second sporulation sequence were also presented leading to the reassignment of *Culicosporella* to the family Amblyosporidae Weiser, 1977.

The ultrastructural characterization of microsporidian species is now considered to be of prime importance for proper taxonomic placement (see Larsson, 1986 for a review of this topic). In this study, the ultrastructural morphology and development of *C. lunata* is described and compared to other heterosporous microsporidia found in

mosquitoes. Additionally, evidence for the occurrence of a third sporulation sequence in adult C. pilosus affords the opportunity to propose a more complete life cycle for C. lunata.

Materials and Methods

Culex pilosus larvae were collected from Gainesville, Florida in the same location as reported by Hazard et al., 1984. Culex pilosus has not been colonized, therefore, field collected material was used in all studies. Larvae with patent fat body infections were isolated for use in EM and light microscopy studies. Adult survivors from field groups, which contained infected specimens, were examined at both the light and EM level. Giemsa and lacto-aceto-orcein (LAO) chromosome staining was done according to the protocol of Hazard et al., 1984.

Infected larval and adult tissues were fixed for 2.5 h at room temperature in 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 0.1% $CaCl_2$ and postfixed in 1% aqueous OsO_4 (w/v). These tissues were dehydrated through an ascending ethanol and acetone series and embedded in Epon-Araldite (Mollenhauer, 1964). Thin-sections were poststained with methanolic uranyl acetate followed by lead citrate (Reynolds, 1963) and photographed at an accelerating voltage of either 50 or 75 kV with a Hitachi HS-8 or H-600 electron microscope.

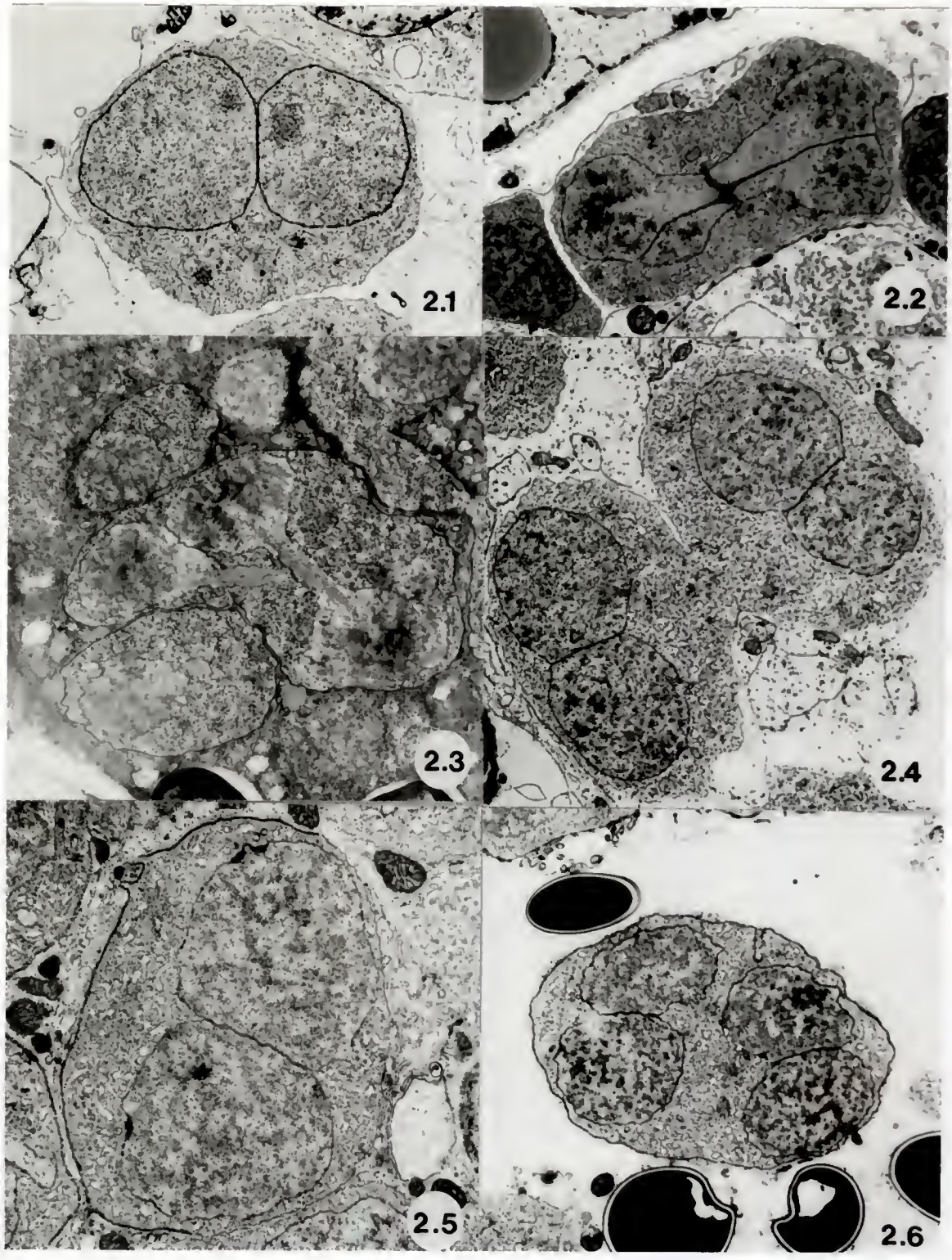
Results

Culicosporella lunata was characterized by two sporulation sequences each producing morphologically distinctive spores in C. pilosus larvae (Hazard et al., 1984). Both sequences were presumably derived from a common diplokaryotic cell (sporont mother cell?) which in one sequence underwent abortive meiosis to sometimes form anomalous meiospores. In the other sequence, thin-walled diplokaryotic spores were produced from diplokaryotic sporogonial plasmodia (Fig. 2.43). Development is presented under two main headings, one detailing the ultrastructure of the two sequences previously described at the light level and another reporting new information on a third sporulation sequence.

Ultrastructure of the Developmental Stages in the Larval Host Presumably Following Transovarial Transmission

Merogony. Spherical or oval diplokaryotic meronts were the earliest stages present in blood and fat body cells of the larval host (Fig. 2.1). The two closely appressed nuclei of the meront formed the diplokaryon which occupied a large portion of the total cell. The nucleoplasm was generally homogeneous, with a darker staining area presumed to represent the nucleolus in each nucleus. Meronts were bound by a unit membrane with the cytoplasm containing a Golgi "reticulum" composed of a system of cisternae, small vesicles and expanded sacs (Figs. 2.1, 2.33). Free

Figures 2.1-2.6. Electron micrographs of selected developmental stages of Culicosporella lunata in larval Culex pilosus involving diplokaryotic sporogonial plasmodia. 2.1) Diplokaryotic meront. x6800; 2.2) Diplokaryotic meront in the process of dividing. x5000; 2.3) Diplokaryotic meront dividing demonstrating the crossing of interpolar microtubules. x8000; 2.4) Final products of division, cytokinesis nearly complete. x5000; 2.5) Transitional form developing into a diplokaryotic sporont as indicated by the partially thickened plasmalemma. Note the doubled spindle plaques on the nuclear envelope of one member of the diplokaryon. x8500; 2.6) Sporogonial plasmodium with two diplokarya and the primordium of the sporophorous vesicle. x5000.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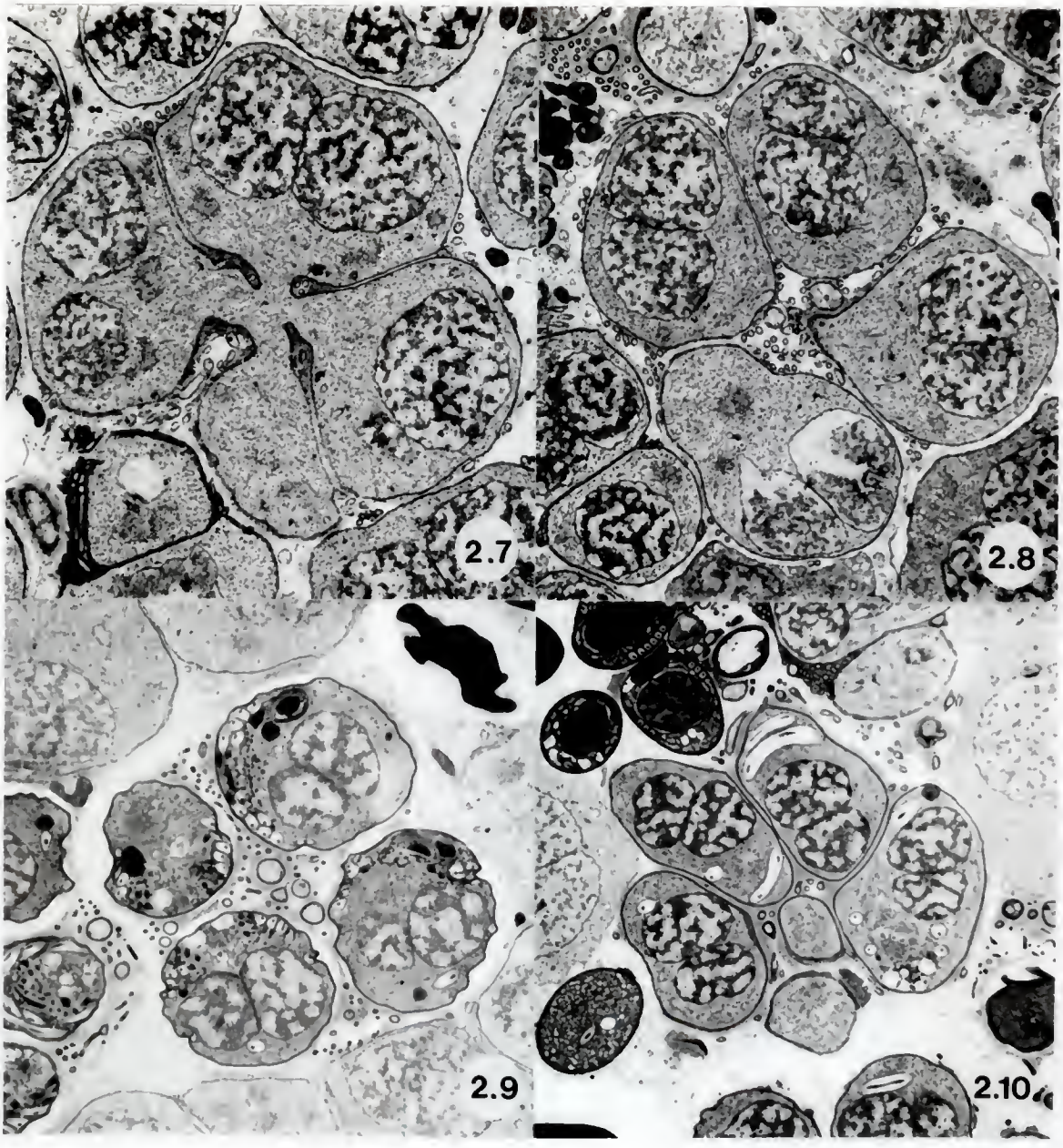
ribosomes and numerous microtubules were dispersed throughout the cytoplasm (Fig. 2.33). Meronts reproduced by binary fission via a process of acentriolar pleuromitosis (Hollande, 1972) in which the nuclear envelope remained intact throughout division. Anaphase of mitosis was represented by 2 dumbbell shaped nuclei (still closely appressed) with interpolar spindle fibers that crossed at the future point of nuclear cleavage (Figs. 2.2, 2.35). This indicated that as karyokinesis proceeded, a twisting event occurred to terminate the process (Figs. 2.3, 2.36) which resulted in the formation of two daughter diplokaryotic meronts (Fig. 2.4).

Transition from merogony to sporogony. At some point, the final diplokaryotic cells of merogony transformed in one of two distinctly different ways. Some underwent repeated karyokinesis with delayed cytokinesis to form diplokaryotic sporogonial plasmodia. Others underwent karyogamy (see Hazard et al., 1984) followed by meiosis. These transformations marked the beginnings of divergent lines of development. Two concurrent sporulation sequences produced two fundamentally different spore types, one haploid and the other diplokaryotic.

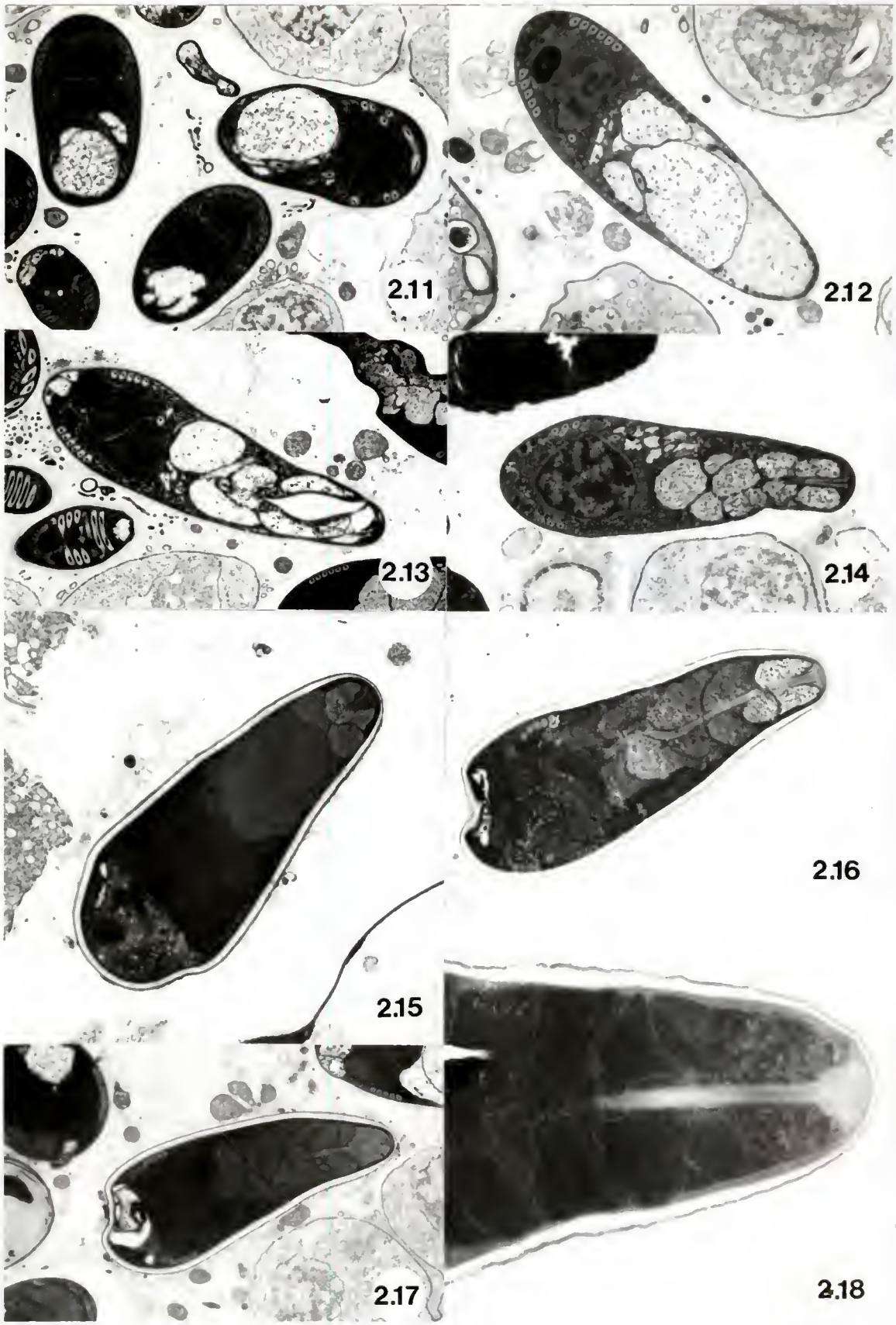
The sporulation sequence that involved sporogonial plasmodia to produce diplokaryotic spores. The initial sporonts could not conclusively be distinguished from meronts due to merogony and sporogony occurring



Figures 2.7-2.10. Electron micrographs of stages of Culicosporella lunata in larval Culex pilosus involving diplokaryotic sporogonial plasmodia. 2.7) Multiple fission of sporogonial plasmodium, cytokinesis nearly complete. x4000; 2.8) Final products of multiple fission. x4000; 2.9) Four sporoblasts within the sporophorous vesicle. x4000; 2.10) Four diplokaryotic sporoblast, elongation and spore morphogenesis underway. x5000.

simultaneously. Stages believed to be early sporonts had the beginnings of a thickened (doubled) plasmalemma (Fig. 2.34) and an extensive golgi "reticulum" composed of cisternae and numerous small vesicles and expanded sacs (Fig. 2.5). Nuclei were commonly observed with doubled spindle plaques and condensed chromatin indicating repeated mitotic divisions (Figs. 2.5, 2.34). During sporogony, a delicate sporophorous vesicle separated from the thickened plasmalemma of the developing sporogonial plasmodium (Figs. 2.6, 2.34). Amorphous material accumulated initially within the sporophorous vesicle (Figs. 2.7, 2.34). As sporulation progressed, tubular material appeared (Figs. 2.8, 2.9), their appearance corresponding with the gradual disappearance of the amorphous material. Diplokaryotic sporogonial plasmodia divided by multiple fission, usually in the form of a rosette (Fig. 2.7), to produce diplokaryotic sporoblasts (Figs. 2.9, 2.10, 2.37, 2.38). During the early stages of sporogenesis, sporoblasts remained spherical and were characterized by a thickened wall and the beginnings of polar tube formation (Figs. 2.9, 2.10). Polar tube formation occurred in two areas of the sporoblast; one area at the anterior end of the future spore where the primordium of the anchoring disc was formed (Figs. 2.9, 2.38); the other in the posterior region by an elaborate system of the Golgi "reticulum" composed of numerous small vesicles, dense elongate cisternae and arrays

Figures 2.11-2.18. Electron micrographs demonstrating the progressive diplokaryotic spore morphogenesis of Culicosporella lunata in larval Culex pilosus. 2.11) Sporoblast in the early stages of morphogenesis, the developing polaroplast a single large vacuole. x5000; 2.12) Developing polaroplast now composed of several large vacuoles. x5000; 2.13) Posterior vacuole now evident, additional large vacuoles in the region of the developing polaroplast. x5000; 2.14) Organization of the developing polaroplast into smaller more numerous vacuoles. x6800; 2.15) Numerous compartments of the developing polaroplast in a helical arrangement. x8500; 2.16) Compartments of polaroplast collapsing dorso-ventrally. x8500; 2.17) Maturing spore with the polaroplast becoming more lamellate. x5000; 2.18) Lamellate polaroplast of the mature diplokaryotic spore. x10,000.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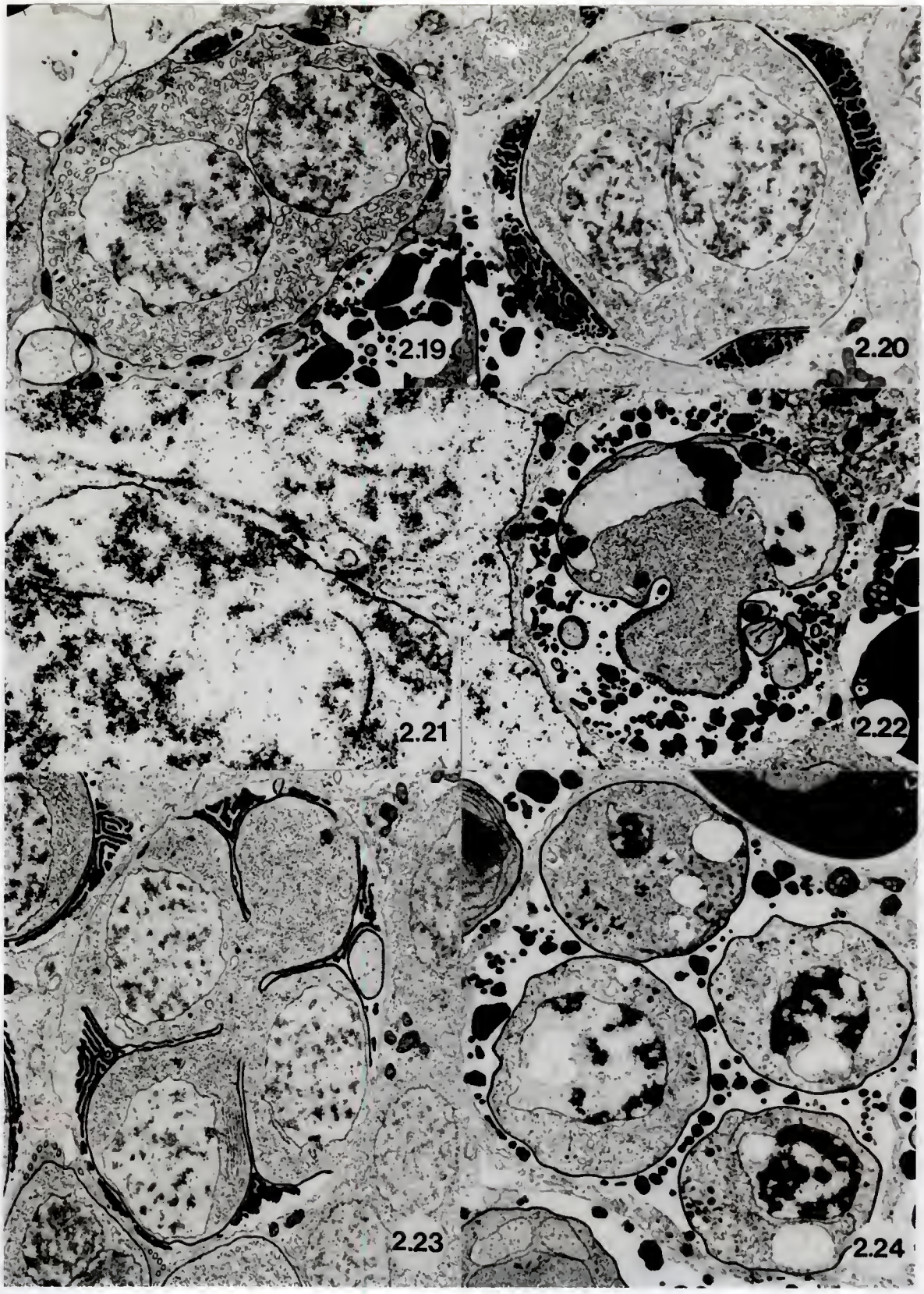
of concentric cisternae (Figs. 2.37, 2.38). Elongation of the sporoblast involved construction of the polaroplast from vesicular protuberances of the polar tube that varied in number and size with development (Figs. 2.11, 2.12, 2.13). These membrane-bound vacuoles gradually accumulated fibrous material, increasing in number while decreasing in size. Eventually, many spherical chambers occupied the anterior two-thirds of the sporoblast (Fig. 2.14). As the spore matured, these chambers were further divided by septa (Figs. 2.15, 2.16, 2.17). In its final form, this organelle was divided into chambers by septa and occupied the anterior half of the spore (Fig. 2.18). The polar tube was isofilar and made 10 to 11 turns about the diplokaryon and the posterior vacuole (Fig. 2.17). A thin exospore and a thicker endospore made up the wall of these lanceolate spores (Fig. 2.18).

The sporulation sequence that followed karyogamy and involved meiosis. Diplokaryotic meronts that underwent karyogamy (see Hazard et al., 1984) entered into a sporulation process that involved meiosis. First indication of this process was the appearance on the plasmalemma of the primordium of the sporophorous vesicle (Fig. 2.41) which developed into small blisters containing granular material (Figs. 2.19, 2.20). Then, the two nuclei of the diplokaryon separated; an act normally followed by the formation of synaptonemal complexes but the observed structures may

Figures 2.19-2.24. Electron micrographs of selected stages of the sporulation sequence involving meiosis to form meiospores of Culicosporella lunata in larval Culex pilosus. 2.19) Early sporont identifiable by the primordia of the sporophorous vesicle. x5000; 2.20) Sporont later in development with axial cores and spindle plaque between the two members of the diplokaryon. x5000; 2.21) Enlargement of that portion of Fig. 2.20 demonstrating axial cores and spindle plaque. x17,000; 2.22) A late sporont with condensed chromosomes, perhaps representing metaphase of meiosis I. x5000; 2.23) Quadrinucleate sporogonial plasmodium. x5000; 2.24) Four early sporoblasts, spore morphogenesis in its earliest stages. x5000.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actually represent axial cores (Figs. 2.20, 2.39, 2.40). A spindle plaque was commonly observed between the two members of the diplokaryon at about this same time (Figs. 2.20, 2.21, 2.39). The axial cores consisted of connected electron dense bead-like bodies (Figs. 2.21, 2.39, 2.40), an apparently unique arrangement (H. C. Aldrich, personal communication). Later, the sporonts appeared to have a large single nucleus where mingling of chromosomes reportedly occurs (Hazard and Brookbank, 1984). The sporophorous vesicle was now fully formed and contained masses of granular material. After metaphase, the first meiotic division proceeded rapidly into the second to produce a quadrinucleate sporont usually in the form of a rosette (Fig. 2.23). A stage in this sequence (Figs. 2.22, 2.42 metaphase?) revealed that the source of the material within the sporophorous vesicle was an expanded cytoplasmic protuberance which extended beneath the nuclei (Fig. 2.42). The outer margins of the protuberance had a spongy appearance, being composed of numerous layers of small vesicles. Some vacuoles, bound by a wall similar to that of the protuberance, and tubular materials were now present within the sporophorous vesicle. In some cases, no further divisions took place while in others a complete or partial third division produced varying numbers of sporoblasts (Figs. 2.24, 2.25). In either case, anomalies in sporogenesis usually occurred and the process aborted.

Figures 2.25-2.26. Electron micrographs of spore morphogenesis of meiospores of Culicosporella lunata in larval Culex pilosus. 2.25) Three young meiospores within the sporophorous vesicle. x10,000; 2.26) Mature meiospore resembling those characteristic of Amblyospora species. x16,000.

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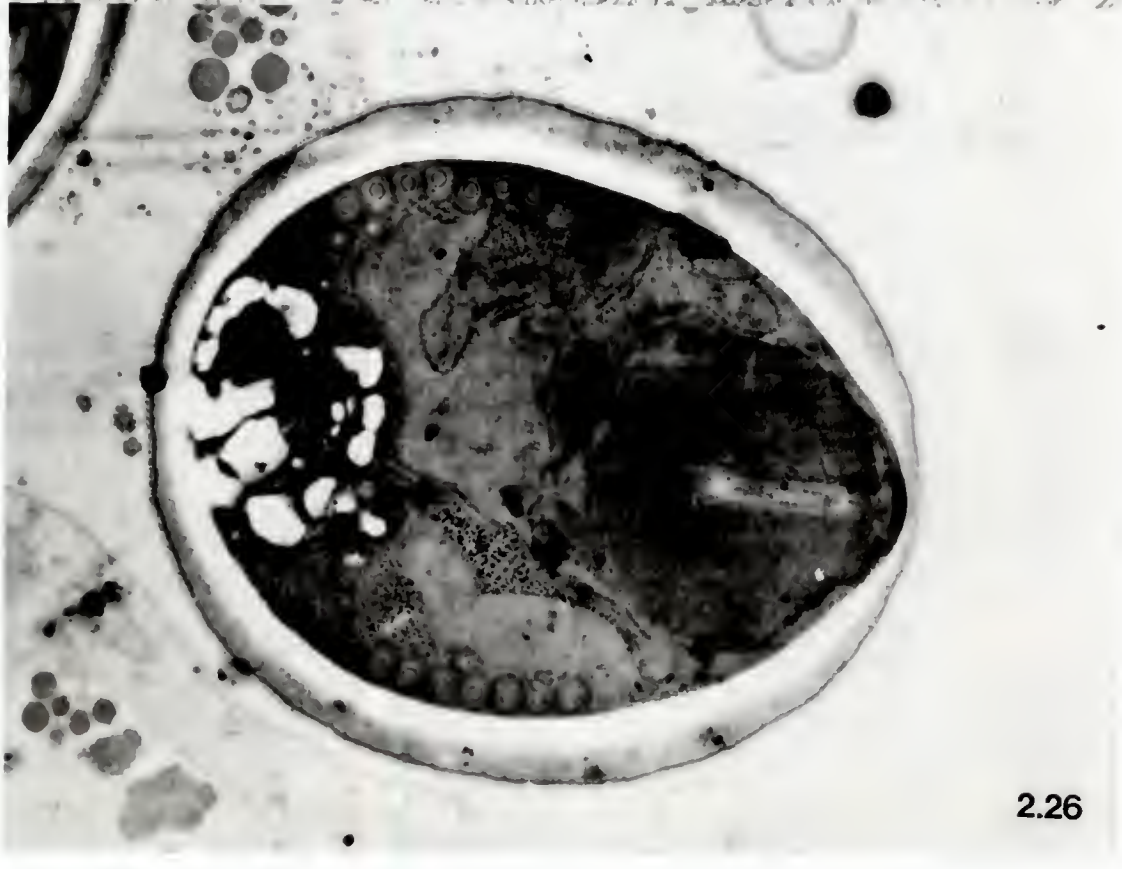

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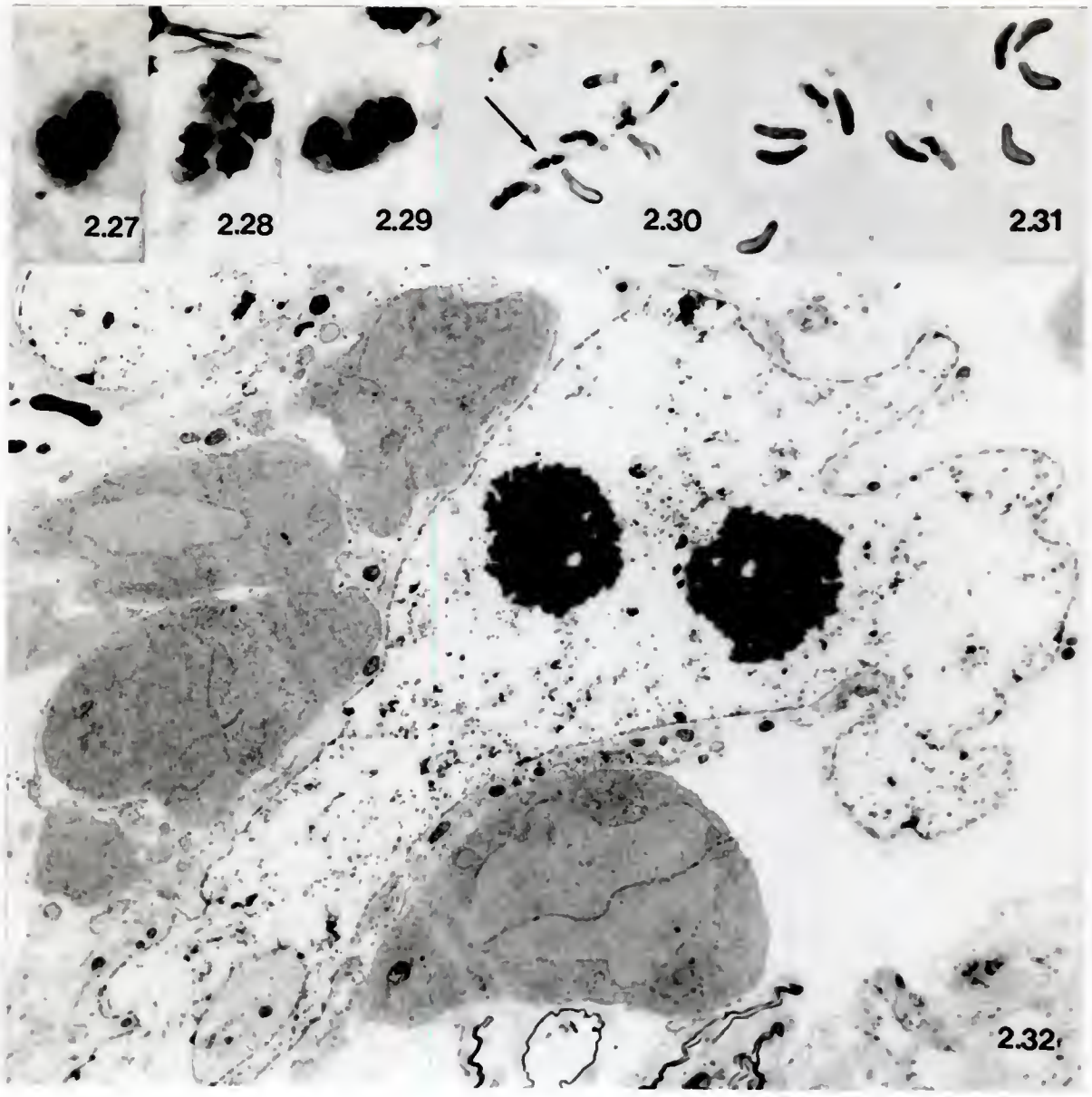
In rare instances, spores ultrastructurally like the meiospores of Amblyospora were formed with a thick wall composed of both an exospore and endospore that was noticeably thinner in the region of the anchoring disc (Fig. 2.26). A lamellar polaroplast and isofilar polar tube that made 11 to 12 turns about the posterior vacuole further characterized the spore (Fig. 2.26).

Observations on the Formation of Nosema-type Spores in Adult Females

Description of the Nosema-type spore. Some adults of C. pilosus (from field collections that contained infected larvae) were found harboring diplokaryotic stages (Figs. 2.27, 2.28, 2.29) and binucleate spores (Figs. 2.30, 2.31) similar to those which are responsible for transovarial transmission reported in Amblyospora, Culicospora and Edhazardia. These spores were binucleate, oblong ovate, often bent and measured 2.35 x 1.38 μ (range 1.64, 3.19 x 1.03, 1.64 ;n=10) in LAO. Electron micrographs of adult tissues (Fig. 2.32) verified the presence of diplokaryotic stages in host cells believed to be oenocytes. Binucleate spores in the adult were not observed ultrastructurally.

Discussion

The ultrastructural observations of this study verified earlier interpretations of the life cycle of C. lunata made with Giemsa and LAO-stained material (Hazard et al., 1984) on the two sporulation sequences that occurred in the larval



Figures 2.27-2.31. Photomicrographs of Culicosporella lunata developmental stages in adult females of Culex pilosus. Giemsa-stained and x2000 excepting 2.30 and 2.31 which are LAO. 2.27) Diplokaryotic meront. 2.28) Karyokinesis of the diplokaryon. 2.29) Meront preparing for cytokinesis. 2.30) Binucleate sporoblast (arrow). 2.31) Group of mature, binucleate spores. Figure 2.32. Electron micrograph of an oenocyte of Culex pilosus infected with diplokaryotic stages of Culicosporella lunata. x10,000.

Figures 2.33-2.34. Electron micrographs of selected developmental stages of Culicosporella lunata in larval Culex pilosus. 2.33) Enlargement of a portion of figure 2.1 demonstrating Golgi (G), microtubules (MT), plasmalemma (PL), and nuclear envelope. x38,000; 2.34) Enlargement of a portion of figure 2.5 demonstrating spindle plaque (SP), plasmalemma (PL), sporophorous vesicle (SV), and amorphous material (arrow). x42,500.

G
PL
MT
NE
2.33
SV
SP
PL
2.34

stages of C. pilosus. The additional observations on binucleate spores in adult C. pilosus suggests that transovarial transmission may also play a role in the developmental cycle of C. lunata (Fig. 2.43).

The golgi "reticulum," in its various morphological forms, was the major cytoplasmic organelle of meronts, sporonts and sporoblasts. Endoplasmic reticulum was noticeably absent during most of the development. Vavra (1965) was perhaps the first to describe from sporoblasts a "primitive" Golgi in microsporidia and suggested a possible function in polar tube formation. Subsequent studies provided additional evidence supporting this function for the Golgi (Sprague and Vernick, 1968; Vavra, 1976b) Vernick et al., 1977, stated "the Golgi reticulum not only concentrates or synthesizes material but, at the same time, contributes a secretory product (as well as some of its membranes) to the wall of the polar filament" (page 98). Evidence presented in this study supports these observations on the role of the Golgi in polar tube formation. Golgi in meronts has only rarely been reported (Andreadis & Hanula, 1987; Becnel et al., 1987, 1989) with no specific function indicated. In C. lunata, the Golgi reticulum appeared to be an active organelle during merogony, becoming progressively more abundant in both the sporont and sporoblasts, functioning to concentrate and secrete cellular materials with a role in both polar tube and polaroplast formation. A

Figures 2.35-2.36. Electron micrographs of selected developmental stages of *Culicosporella lunata* in larval *Culex pilosus*. 2.35) Enlargement of figure 2.2 demonstrating the crossing of spindle fibers (SF). x17,000; 2.36) Enlargement of figure 2.3 demonstrating the crossing of spindle fibers (SF). x19,000.

2.35

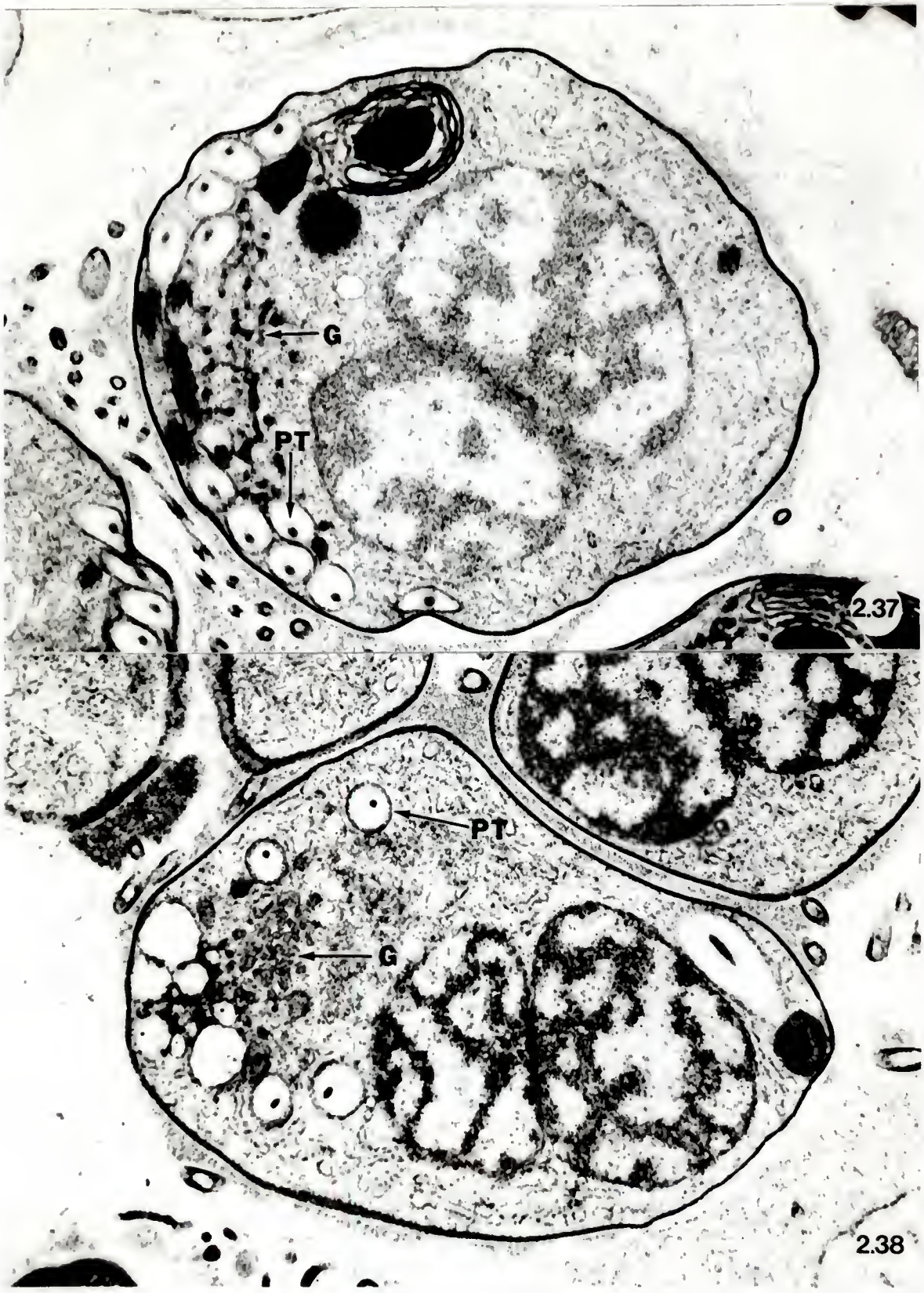
2.36

similar function for the Golgi has been reported in Glugea stephani (Jensen and Wellings, 1972).

Replicated spindle plaques with their associated polar vesicles were common, indicating that the completion of one division was rapidly followed by another. These spindle plaques apparently migrated to opposite ends of each nucleus with the spindle fibers forming a line parallel with the line of apposition of the two nuclei. The nuclear envelope remained intact throughout the division. The closely appressed nature of the two members of the diplokaryon was maintained as each nucleus elongated and began cytokinesis. Interpolar microtubules persisted throughout karyokinesis and crossed at the future point of cleavage. This indicated that the nuclei twisted as they separated from one another with the resulting final products mirror images. Previous studies have presented information on the division of the diplokaryon, some indicating a crossing of spindle fibers (Debaisieux, 1919; Hazard et al., 1984). Ultrastructural indications of this twisting phenomenon have been reported (Andreadis and Hall, 1979a, Fig. 30; Becnel et al., 1987, Fig. 23) but this conclusive documentation is new.

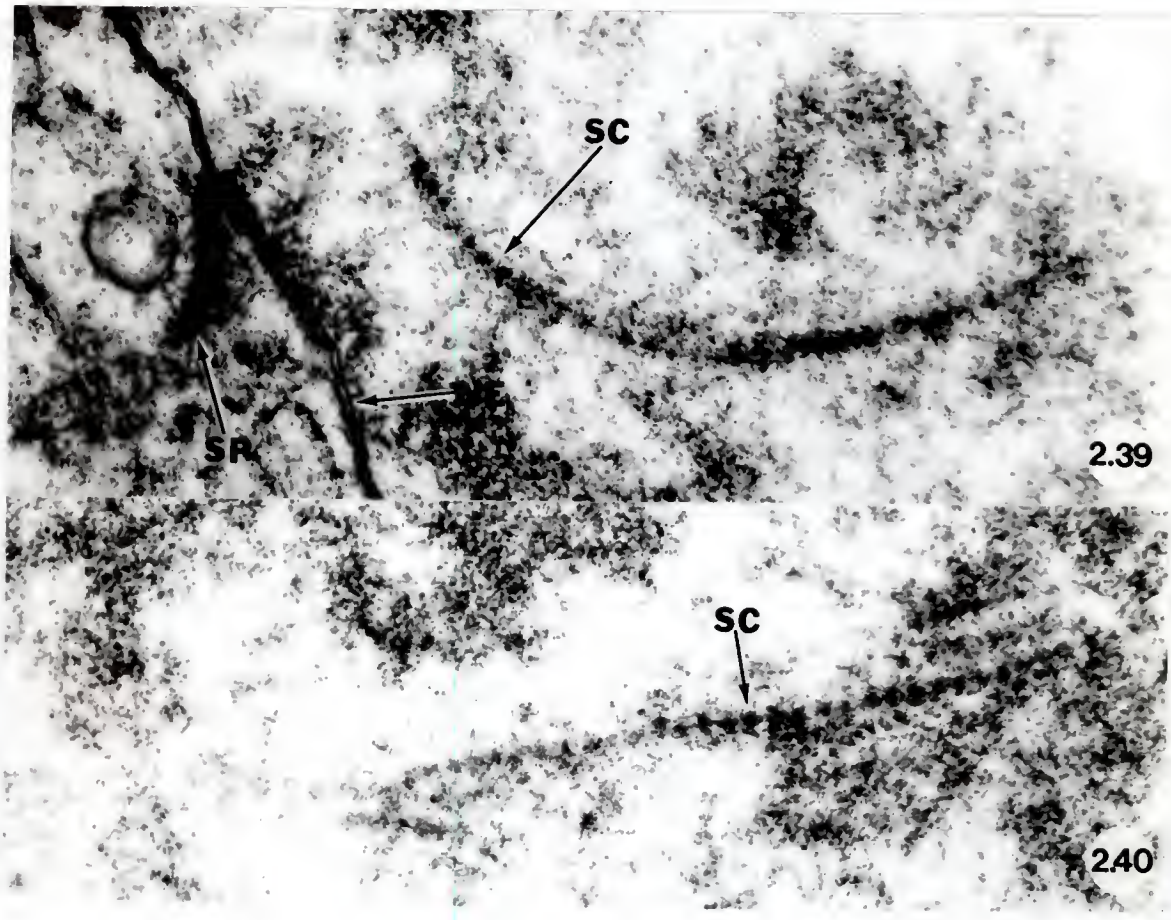
Vavra (1976a) proposed two possible modes of division of the diplokaryon, one producing "daughter" cells the other producing "cousin" cells with the latter alternative more likely. This terminology has caused some confusion with researchers in that his "cousin" cells are identical to the

Figures 2.37-2.38. Electron micrographs of selected developmental stages of Culicosporella lunata in larval Culex pilosus. 2.37) Enlargement of sporoblast in figure 2.9 demonstrating the Golgi (G) and polar tube (PT). x18,000; 2.38) Enlargement of sporoblast in figure 2.10 demonstrating the Golgi (G) and polar tube (PT). x22,500.


G
PT
2.37
PT
G
2.38

parent while the "daughter" cells are different (his rationale apparently being that the original diplokaryon consisted of "cousin" nuclei). Toguebaye and Marchand (1984), using the terminology of Vavra, reported that in Nosema henosepilachnae division of the diplokaryon resulted in "cousins" (division products like the parent). Streett and Henry (1987) found that, in N. cuneatum, karyokinesis of the diplokaryon resulted in "daughter" diplokarya each with one member of the original diplokaryon (therefore like the parent). If one considers the diplokaryon as a genetic unit (even though the two nuclei may or may not be identical), the division products each consist of a genetic unit like the parent. Confusion stems by the deviation from the commonly accepted use of "daughter" cell as one genetically like the parent cell. Since this and the previous studies agree that the 2 cells produced are genetically like, the term "daughter" is more appropriate.

These studies have presented evidence that agree with previous findings on the division of the diplokaryon, with the addition of the twisting mechanism (Vavra, 1976a; Toguebaye & Marchand, 1984; Streett & Henry, 1987). It has been clearly documented that division of the diplokaryon involves the twisting of the elongated nuclei during karyokinesis, this playing a role in the completion of the process. This twisting event resulted in apparently the same division products (daughter cells) as if this event had



Figures 2.39-2.40. Electron micrographs of selected developmental stages of Culicosporella lunata in larval Culex pilosus. 2.39) Enlargement of a portion of the nucleus in figure 2.21 showing an axial core (SC), spindle plaque (SP), and the nuclear envelope (NE). x22,000; 2.40) Enlargement of a portion of the nucleus in figure 2.21 showing an axial core (SC). x22,500.

not occurred. It may be speculated that this twisting is related to a more energy efficient method for the completion of karyokinesis of the diplokaryon in the microsporidia.

In species of Amblyospora with a successful meiotic sequence that produces meiospores, synaptonemal complexes are formed with a central element and two lateral elements (Chapter III, Fig. 3.21). In C. lunata, the presence of axial cores rather than synaptonemal complexes may explain the abortive meiosis. A sporulation defective mutant of Saccharomyces was shown to form axial cores rather than synaptonemal complexes during meiosis (Bennett, 1983). It was concluded that premeiotic DNA synthesis is required for central element assembly in synaptonemal complexes. Investigations of premeiotic events in C. lunata and documentation of axial cores in other species with abortive meiosis are necessary to determine the reason for the failure of meiosis.

The dominant sporulation sequence in C. lunata produced diplokaryotic spores from sporogonial plasmodia. This sequence and the resulting spores were similar to those reported for C. magna (Becnel et al., 1987) and E. aedis (Becnel et al., 1989) except that the spores produced in these latter species were haploid as a result of nuclear dissociation. These spores are also similar in function, responsible for horizontal transmission to their mosquito hosts. The spores of some species of Amblyospora

Figures 2.41-2.42. Electron micrographs of selected developmental stages of Culicosporella lunata in larval Culex pilosus during meiosis. 2.41) Enlargement of a portion of the outer membranes of the sporont in figure 2.19 showing the plasmalemma (PL) and the sporophorous vesicle (SV). x100,000; 2.42) Enlargement of the sporont in figure 2.22 showing spindle fibers (SF) and the ventral protuberance. x10,500.

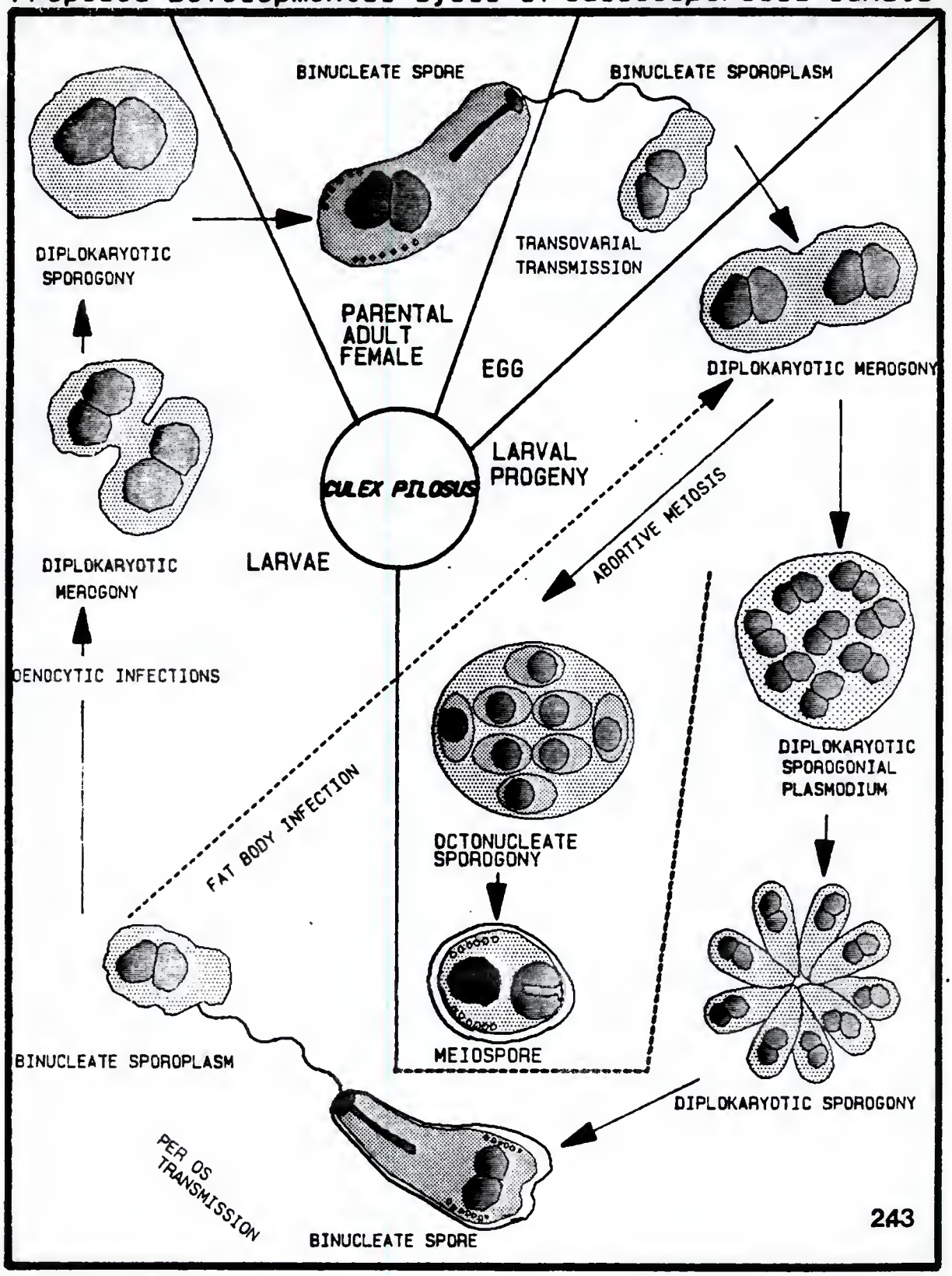
responsible for horizontal transmission to the mosquito are also similar except that they are formed in a copepod intermediate host (Sweeney et. al., 1985, 1988; Andreadis, 1988a).

The diplokaryotic stages and the Nosema-like binucleate spores found in the adult females of C. pilosus suggests that transovarial transmission may play a role in the developmental cycle of C. lunata. Direct horizontal and vertical transmission data are lacking to conclusively prove that the binucleate spores found in the adult represent C. lunata. However, based upon previously documented developmental cycles for other heterosporous microsporidia (Andreadis, 1988a; Sweeney et al., 1988; Becnel et al., 1989) and evidence presented here, a tentative developmental cycle can be proposed.

Proposed developmental cycle for C. lunata. Binucleate spores are formed in oenocytes of the adult female responsible for transovarial transmission to the successive mosquito generation (Fig. 2.43). Two sporulation sequences occur concurrently in the fat body of progeny, both with spores in sporophorous vesicles; one that involves meiosis and usually aborts but occasionally produces meiospores like those of Amblyospora; the other, the dominant one, involves diplokaryotic sporogonial plasmodia and ends with lanceolate, diplokaryotic spores of a form similar to those of C. magna and E. aedis but without the involvement of

Figure 2.43. Diagramatic representation of the proposed developmental cycle of Culicosporella lunata in the mosquito Culex pilosus.

Proposed Developmental Cycle of *Culicosporella lunata*



nuclear dissociation of the diplokaryon. This diplokaryotic spore is responsible for horizontal transmission when ingested by C. pilosus larvae. It is also conceivable that some transovarially infected progeny survive to the adult stage and that infected females transmit the disease transovarially as found in E. aedis (Becnel et al., 1989). Diplokaryotic merogony following horizontal transmission (per os) can apparently lead to different ends. The fat body may be directly invaded where the two concurrent sporulation sequences occur (Hazard et al., 1984). Alternatively, some meronts may enter oenocytes where in the adult female, binucleate spores are formed to transovarially transmit the parasite to progeny. Based on the flexibility reported in E. aedis (Becnel et al., 1989) all alternatives may occur, playing important roles in parasite maintenance.

CHAPTER III
FORMAL DESCRIPTION OF A NEW MICROSPORIDIAN PARASITE IN THE AUSTRALIAN MOSQUITO *Culex halifaxi*

Introduction

During a survey of Australian mosquitoes for pathogenic microorganisms, E. I. Hazard and A. W. Sweeney found several larvae of the predacious mosquito *Culex halifaxi* which were found harboring a microsporidium. First observations revealed the presence of eight, oval spores within a sporophorous vesicle, implicating this species as a member of the genus *Amblyospora*. Subsequent investigation documented the presence of an overlooked second, concurrent sporulation sequence. This study presents the developmental sequences and morphological features of this new and unusual microsporidium of *C. halifaxi*. Similarities and differences between this new microsporidium and other heterosporous microsporidia in mosquitoes are examined to determine its proper taxonomic placement.

Materials and Methods

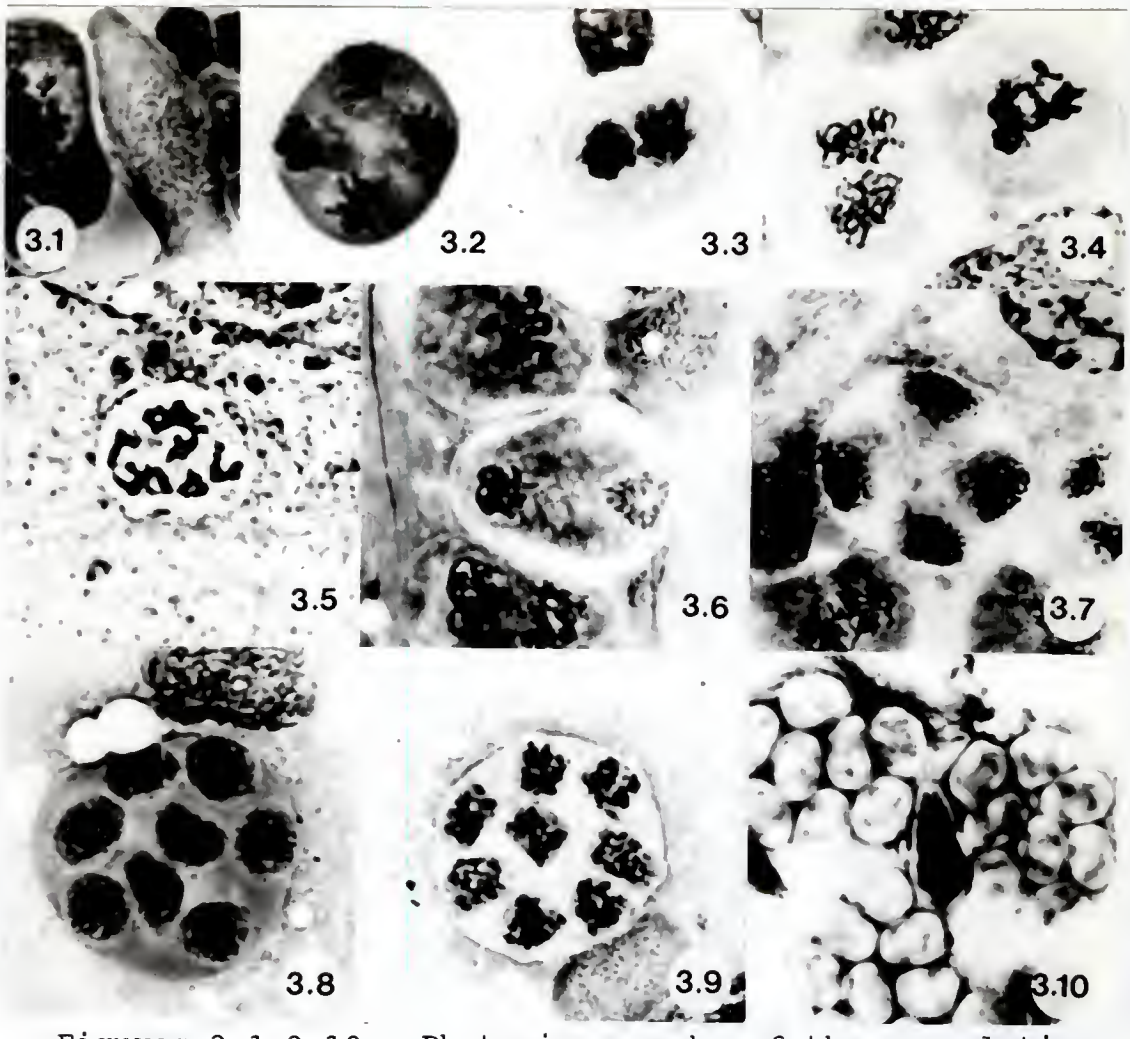
Culex halifaxi Theobald 1903 larvae were collected in March, 1983, from a shaded rain forest stream (clear, slow flowing water) at the foot of the Brown Range near Cowley Beach, Queensland, in Australia. Six of approximately 40 larvae were determined to have patent fat body infections of

an unknown microsporidium. Giemsa and Heidenhain-stained material were prepared according to previously described protocols (Hazard et al., 1984). Tissues for electron microscopy were fixed overnight in 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.3), washed in buffer and held in the cold until returning to the laboratory. Larvae were postfixed in 1% Daltons Chrome Osmium in the cold for three hours and then replaced with 0.5% aqueous uranyl acetate for 1 hour. The remainder of the processing followed the protocol previously described in Chapter II.

Results

This microsporidium proved to be a heterosporous species with two concurrent sporulation sequences (Fig. 3.47). Both began with diplokaryotic meronts and ended with haploid spores of two types. Haplosis in one sequence involved meiosis to produce meiospores similar to those of Amblyospora (Hazard and Oldacre, 1975); in the other sequence it was by nuclear dissociation of the diplokaryon to produce spores similar to Culicospora (Becnel et al., 1987).

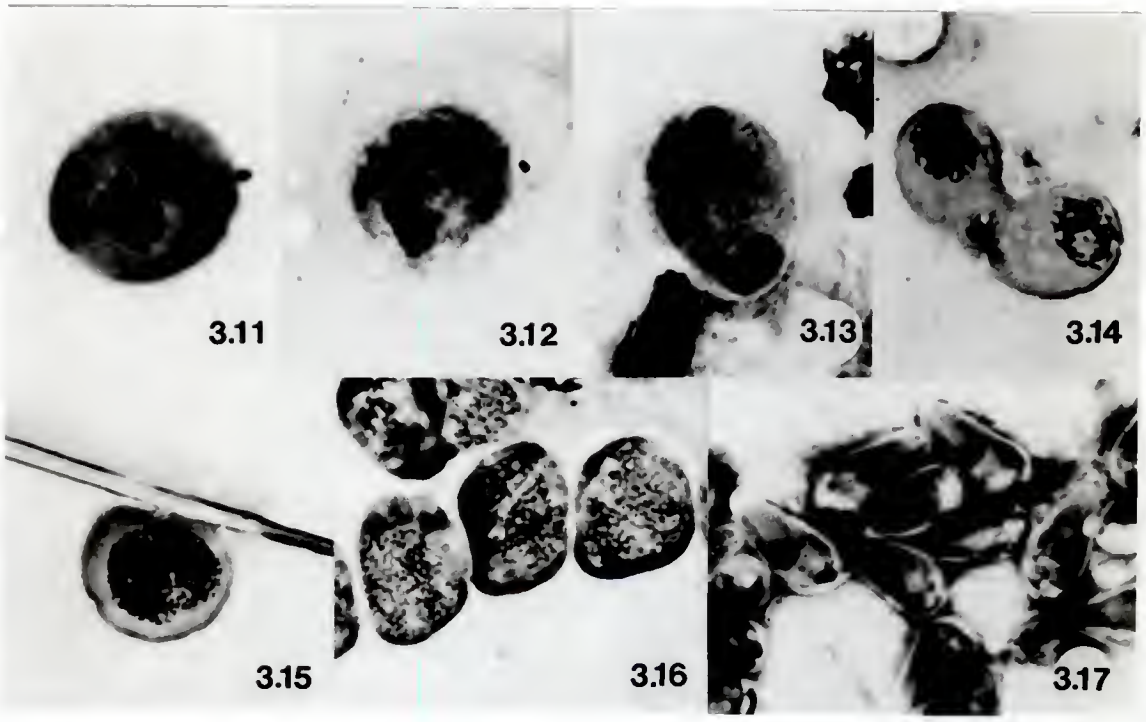
Merogony and transitional forms. Stages interpreted as meronts were diplokaryotic, round in shape and reproduced by binary fission (Figs. 3.18, 3.31). Some meronts apparently underwent karyogamy, thereby becoming zygotes, followed by restoration of the diplokaryon to become sporonts. These sporonts proceeded directly into the sporulation sequence



Figures 3.1-3.10. Photomicrographs of the sporulation sequence of Amblyospora sp. in larval Culex halifaxi that involves meiosis and ends with meiospores. All Giemsa-stained and x2000 excepting Fig. 3.5 which is Heidenhain-stained. 3.1) Fusiform stage with what appears to be a large, single nucleus (synkaryon?). 3.2) Diplokaryotic stage dividing, perhaps the final division prior to the onset of meiosis. 3.3) A sporont with the sporophorous vesicle indistinctly evident. 3.4) Slightly more advanced sporonts, the one on the left with chromosomes of each member of the diplokaryon condensed; the one on the right with a single nucleus due to the coalescence of the two members of the diplokaryon. 3.5) Chromosomes in a condensed state. 3.6) Binucleate stage resulting from the first meiotic division. 3.7) Tetranucleate sporogonial plasmodium after the second meiotic division. 3.8) Quadrinucleate sporogonial plasmodium. 3.9) Eight sporoblasts within a sporophorous vesicle. 3.10) Groups of eight mature meiospores within sporophorous vesicles. Note the pyriform spore from nuclear dissociation.

that involved meiosis. Haplosis in other meronts was by nuclear dissociation to form haploid sporonts.

Sporulation sequence that involved meiosis. Fusiform stages that may represent transitional forms were observed in both Giemsa and EM preparations (Figs. 3.1, 3.19). These sometimes appeared to be uninucleate in Giemsa while in EM they were diplokaryotic and perhaps divided once prior to meiosis (Fig. 3.2). The nucleus characteristically had dispersed clumps of chromatin with spindle plaques commonly observed on the envelope. The cytoplasm contained small arrays of endoplasmic reticulum (Fig. 3.40) and free ribosomes and was bound by a delicate plasmalemma (Figs. 3.19, 3.40). The first indications of meiosis were a doubled plasmalemma and an extensive Golgi reticulum in the cytoplasm (Figs. 3.20, 3.41). The small vesicles of the Golgi were now tightly clustered, sometimes closely associated with the nuclear envelope (Fig. 3.42). Rough endoplasmic reticulum dominated the cytoplasm of the sporonts at this time (Figs. 3.21, 3.41, 3.42). Cells entering meiosis were identifiable in Giemsa-stained material by a halo about the sporont representative of the sporophorous vesicle (Fig. 3.3) The two nuclei of the diplokaryon remained closely appressed with the chromatin appearing less dispersed. As the process proceeded, the sporophorous vesicle separated from the plasmalemma within which accumulated dense masses of granular material (Figs.

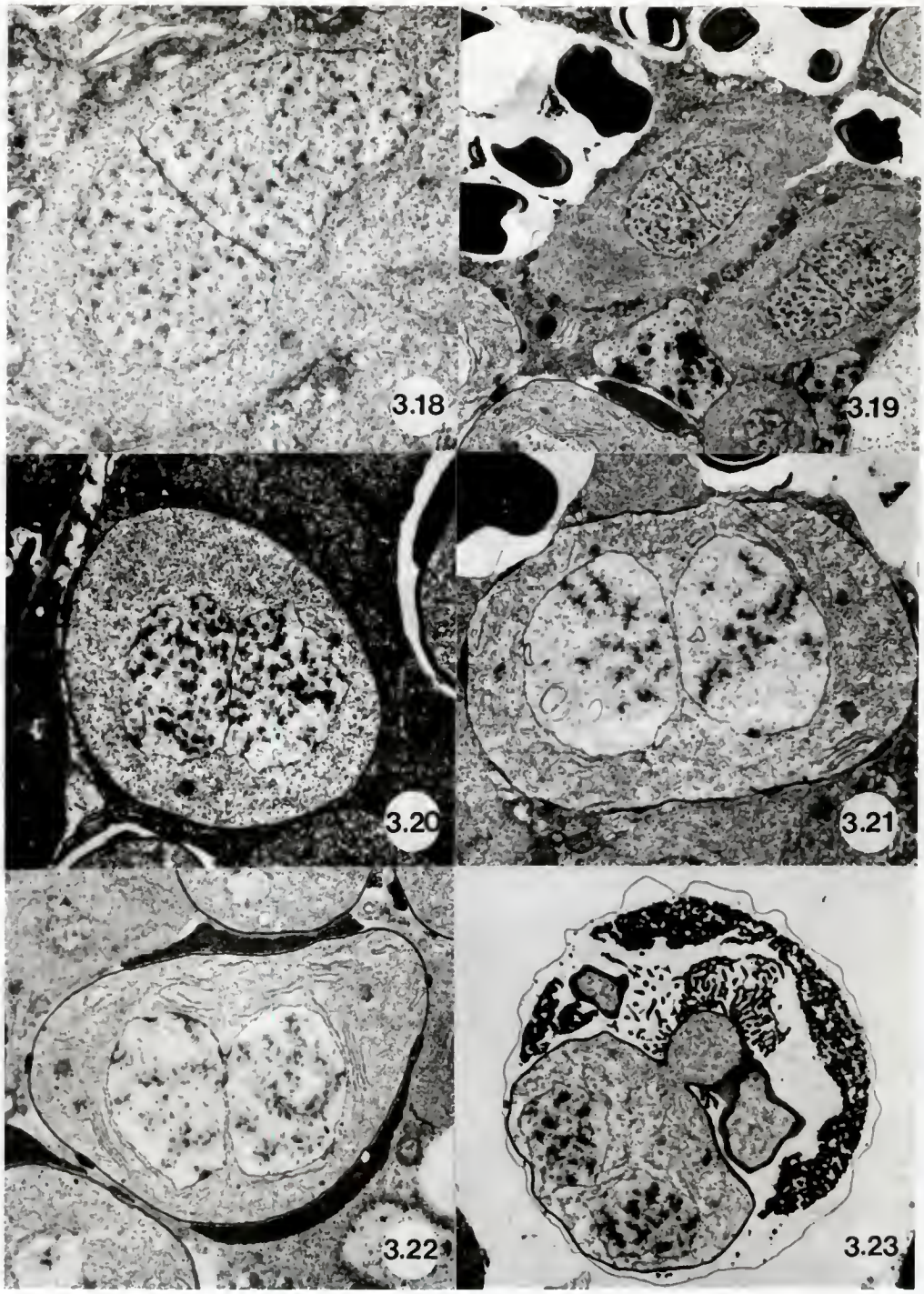


Figures 3.11-3.17. Photomicrographs of the sporulation sequence of Amblyospora sp. in larval Culex halifaxi that involves nuclear dissociation and ends with uninucleate, pyriform spores. All Giemsa-stained and x2000. 3.11) Diplokaryotic sporont with the nuclei only loosely associated. 3.12) Nuclear dissociation. 3.13) Stage after nuclear dissociation with the two nuclei at opposite ends of the cell. 3.14) Sporont undergoing cytokinesis. 3.15) Uninucleate sporont, a product of nuclear dissociation. 3.16) Group of late sporonts. 3.17) Uninucleate, pyriform spores as a result of nuclear dissociation.

3.21, 3.22, 3.42). A spindle plaque was now located between the two members of the diplokaryon with the 2 nuclei only loosely associated (Fig. 3.22). Synaptonemal complexes were present in these nuclei as well as membrane bound vacuoles with electron dense inclusions. Similar vacuoles were found in the cytoplasm which, in addition to the golgi reticulum, now contained numerous arrays of rough endoplasmic reticulum. Metaphase (Figs. 3.4, 3.24) was rapidly followed by the first and second meiotic divisions. Cytokinesis was normally delayed with the production of binucleate (Figs. 3.6, 3.25), tetranucleate (Figs. 3.7, 3.26), and finally octonucleate plasmodia (Fig. 3.8). In some binucleate sporonts, a protuberance was noted with tubular materials attached (Figs. 3.23, 3.45). In later stage sporonts, the granular products appeared to organize into sheets which were often in a close, parallel relationship with the developing spore wall of the sporonts and sporoblasts (Figs. 3.27, 3.28). As the spores matured, the material within the sporophorous vesicle disappeared (Figs. 3.9, 3.29). Eight meiospores, characteristic of Amblyospora, were the end product of this process (Figs. 3.10, 3.30). These spores were broadly oval, thick-walled and measured 4.30 ± 0.04 x 3.44 ± 0.03 μ (mean$\pm$SE) in Heidenhain-stained material.

The sporulation sequence that involved nuclear dissociation. This sporulation sequence began when the two nuclei of the diplokaryon dissociated to form uninucleate

Figures 3.18-3.23. Electron micrographs of selected stages in the sporulation sequence of Amblyospora sp. in larval Culex halifaxi involving meiosis and ending with meiospores. 3.18) Diplokaryotic meront. x8500; 3.19) Fusiform diplokaryotic stages prior to the beginning of meiosis. x4000; 3.20) Early sporont distinguished by the thickened plasmalemma. x5000; 3.21) Later sporont, the primordia of the sporophorous vesicle evident as blisters on the plasmalemma. Synaptonemal complexes present and attached to the nuclear envelope. x5000; 3.22) Sporont with granular material within the sporophorous vesicle. Synaptonemal complexes still evident with a spindle plaque now located between the two members of the diplokaryon. x5000; 3.23) Sporont, protuberance with tubular material. x5000.


3.18
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3.21
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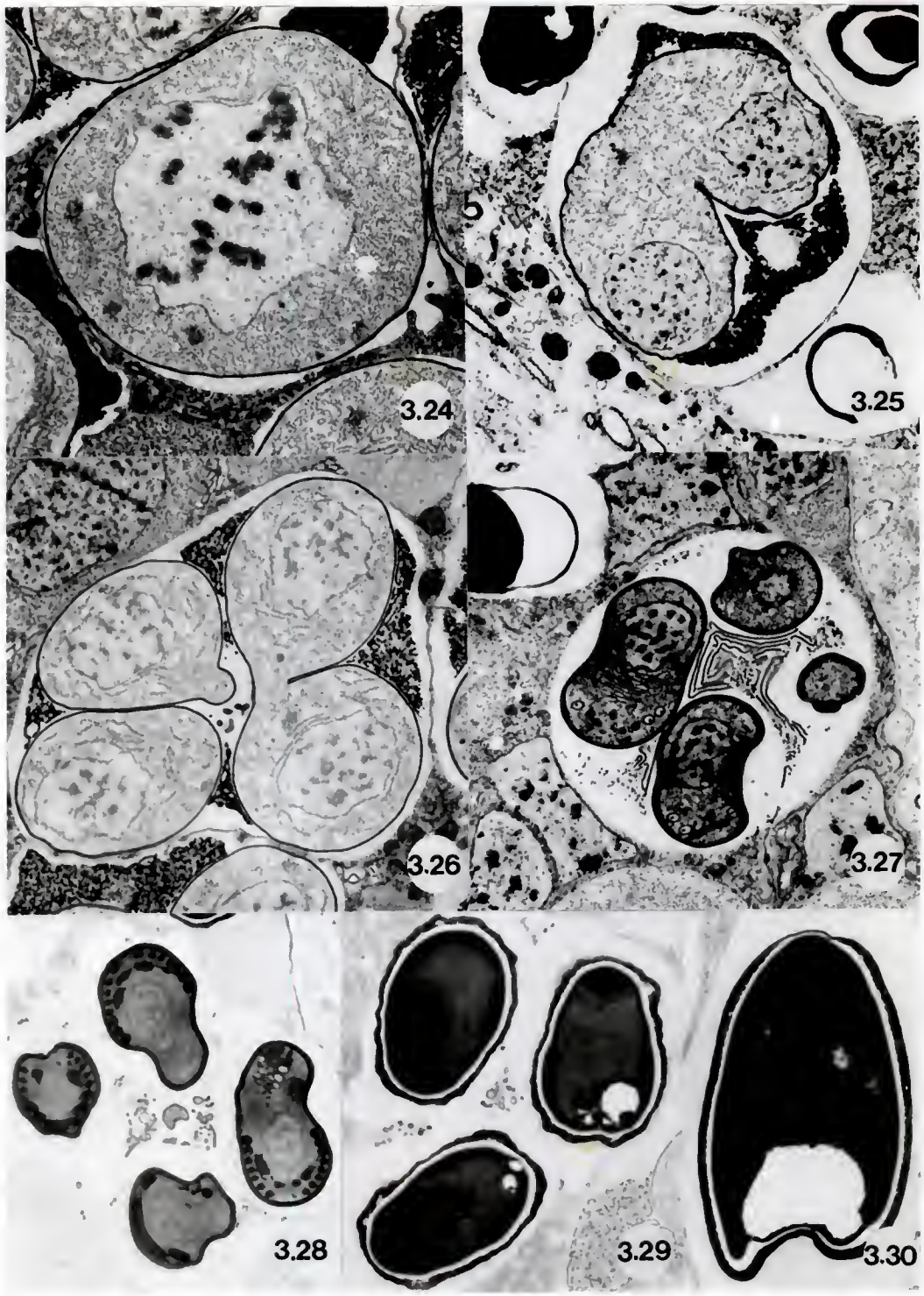
3.23

sporonts (Figs. 3.11-3.14, 3.32). In Giemsa-stained preparations, these stages had densely stained cytoplasm and compact nuclei (Figs. 3.15, 3.16). Sporonts apparently transformed directly into uninucleate spores without further divisions. Elongation of the sporont was an early indication of spore morphogenesis (Figs. 3.33, 3.34). Otherwise, these spores were characteristic of those formed in E. aedis (Becnel et al., 1989), C. magna (Becnel et al., 1987) and Amblyospora (Andreadis, 1985a; Sweeney et al., 1985). A delicate, nonpersistent sporophorous vesicle, within which amorphous material accumulated, enveloped the sporoblasts (Figs. 3.35, 3.36). The pyriform spores developed in a manner similar to that reported for C. lunata (Chapter II). An extensive polaroplast was constructed (Fig. 3.37) with the involvement of the Golgi reticulum (Fig. 3.43). A helical arrangement of septate chambers formed the polaroplast with membranes derived from the polar tube (Figs. 3.38, 3.44) and occupied the anterior two thirds of the spore. The polar tube made 12 to 13 turns in the posterior region of the thin-walled, uninucleate spores (Figs. 3.39, 3.46). Mature uninucleate spores were lanceolate and measured $8.47 \pm 0.12 \times 3.72 \pm 0.05$ μ in Heidenhain-stained material (Fig. 3.17) .

In an adult female C. halifaxi examined (from the collection that contained infected larvae) no diplokaryotic cells or binucleate spores were present. However, stages

Figures 3.24-3.30. Electron micrographs of selected stages in the sporulation sequence of Amblyospora sp. in larval Culex halifaxi involving meiosis and ending with meiospores. 3.24) Sporont at metaphase. x8000; 3.25) Binucleate sporont within a fully formed sporophorous vesicle. x5000; 3.26) Quadrinucleate sporont, cytokinesis incomplete. x8000; 3.27) Sporoblasts within the sporophorous vesicle, the granular material appearing sheet-like. x5000; 3.28) Mature sporoblasts within the sporophorous vesicle. x5000; 3.29) Three immature spores, granular material absent. x8000; 3.30) Mature meiospore. x12,000.


3.24
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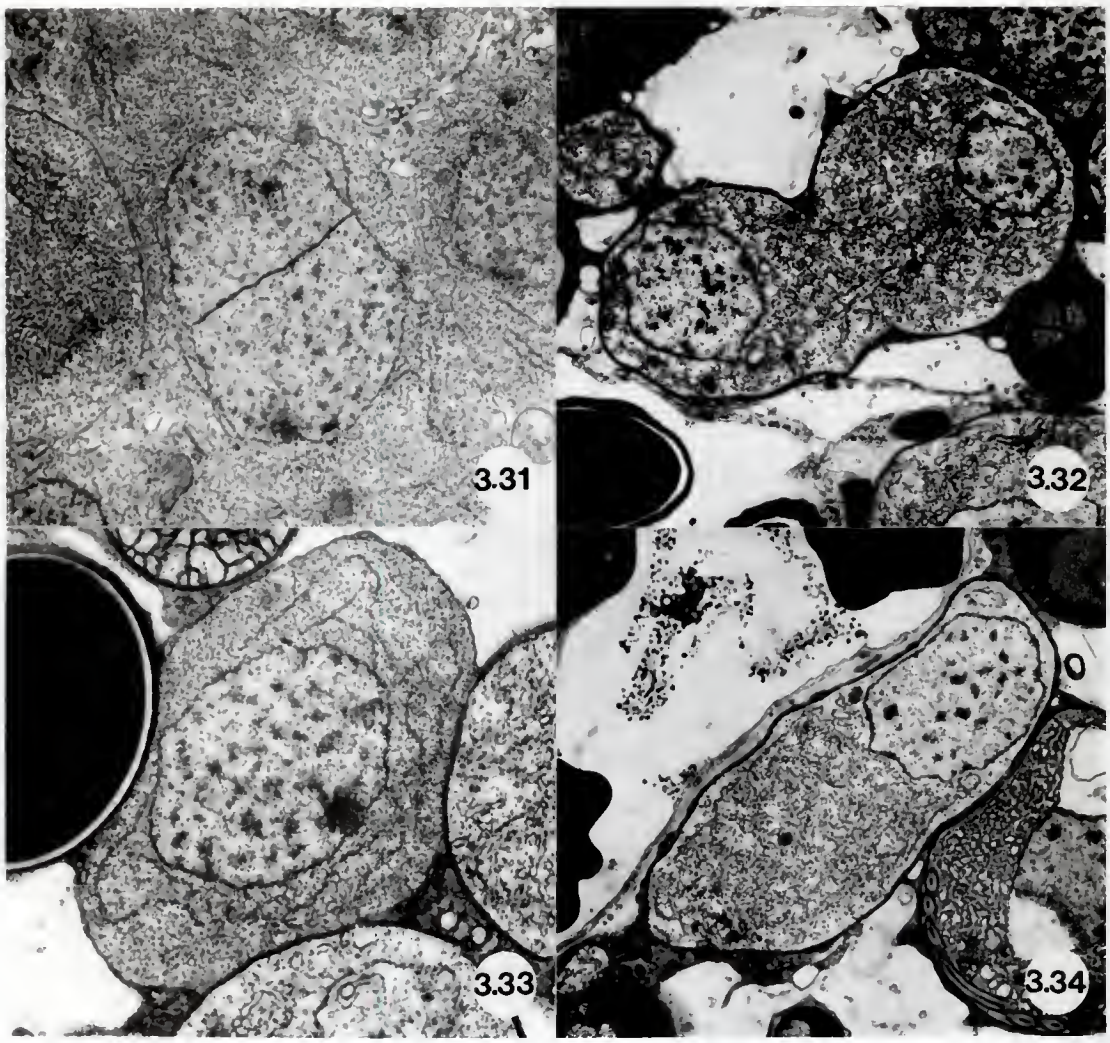
3.30

were found that were characteristic of the horizontally induced sequence in E. aedis, C. magna and Amblyospora. In these latter species, uninucleate stages of this type (gametes) undergo a developmental sequence that involves plasmogamy and nuclear association to form diplokaryotic cells. Transformation of these cells give rise to binucleate spores in the female adult responsible for transovarial transmission.

Discussion

In a previous survey, the only organisms found infesting C. halifaxi were two species of Vorticella (Debenham, 1988). Becnel (1986) reported the discovery of this new microsporidium infecting C. halifaxi and designated it as a species of Amblyospora. Subsequently, it was reported that this species had 3 sporulation sequences in one host with the meiotic sequence dominant, successfully ending with meiospores (Becnel et al., 1989). This study has confirmed earlier reports and has documented the necessary information to characterize this Amblyospora sp. and consider its affinities to other heterosporous microsporidia in mosquitoes.

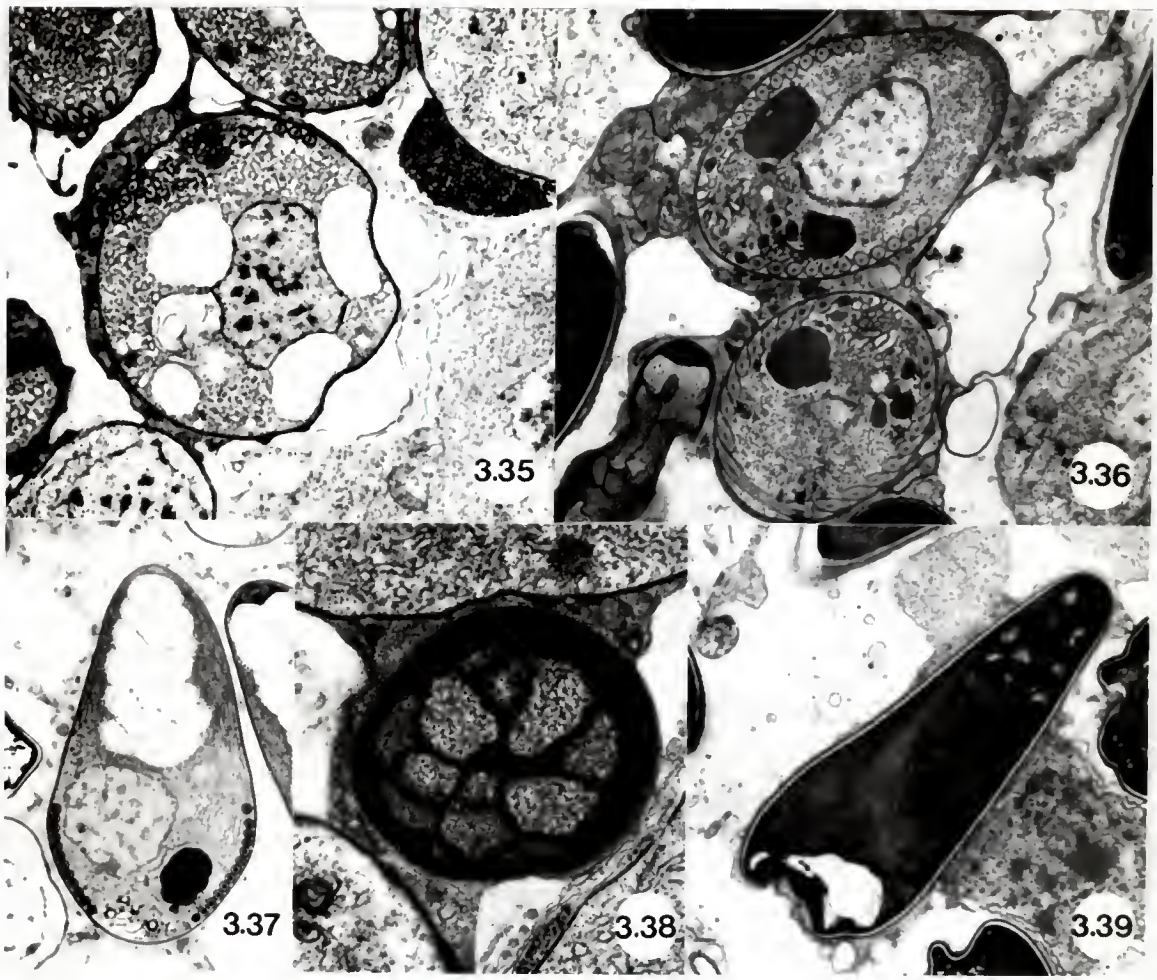
The sporulation sequence that involved meiosis and the morphology of the resulting spores of this Amblyospora sp. were similar to those previously reported for several heterosporous microsporidia (Andreadis, 1988a; Becnel et al., 1986, 1989; Sweeney et al., 1988) without any



Figures 3.31-3.34. Electron micrographs of selected stages in the sporulation sequence of Amblyospora sp. in larval Culex halifaxi involving nuclear dissociation and ending with pyriform, uninucleate spores. 3.31) Diplokaryotic meront. x10,000; 3.32) Sporont undergoing nuclear dissociation within a delicate sporophorous vesicle. x8000; 3.33) Uninucleate sporont, a product of dissociation. x6000; 3.34) Later sporont, becoming elongate, the plasmalemma thickening. x10,000; 3.35) Uninucleate sporoblast, the sporophorous vesicle still closely applied to the plasmalemma within which are amorphous materials. x6800.

significant variations. Perhaps the most difficult stage to document ultrastructurally in this and previous studies has been the synkaryon (zygote). Light level documentation has demonstrated karyogamy prior to meiosis in an Amblyospora sp. (Hazard and Brookbank, 1984), C. lunata (Hazard et al., 1984), and E. aedis (Becnel et al., 1989); however, stages interpreted as the synkaryon have only been reported ultrastructurally in C. magna (Becnel et al., 1987). In C. magna, karyogamy occurred prior to the formation of binucleate spores in the adult female mosquito. The developmental cycle of C. magna has been thoroughly examined (Becnel et al., 1987) and meiosis has not been observed. This is perhaps the reason for the occurrence of karyogamy at a point in the developmental cycle that differs from the other species. Regardless of its position in the cycle, karyogamy appears to be a very rapid process which could account for the lack of ultrastructural evidence in species of Amblyospora.

The sporulation sequence that involved nuclear dissociation and the resulting spores for this Amblyospora sp. were similar to those reported for C. magna (Becnel et al., 1987) and E. aedis (Becnel et al., 1989). The present species differed from C. magna and E. aedis in that once nuclear dissociation occurred, the resulting uninucleate sporonts either transformed directly into spores or underwent binary fission to form two spores. In both E.

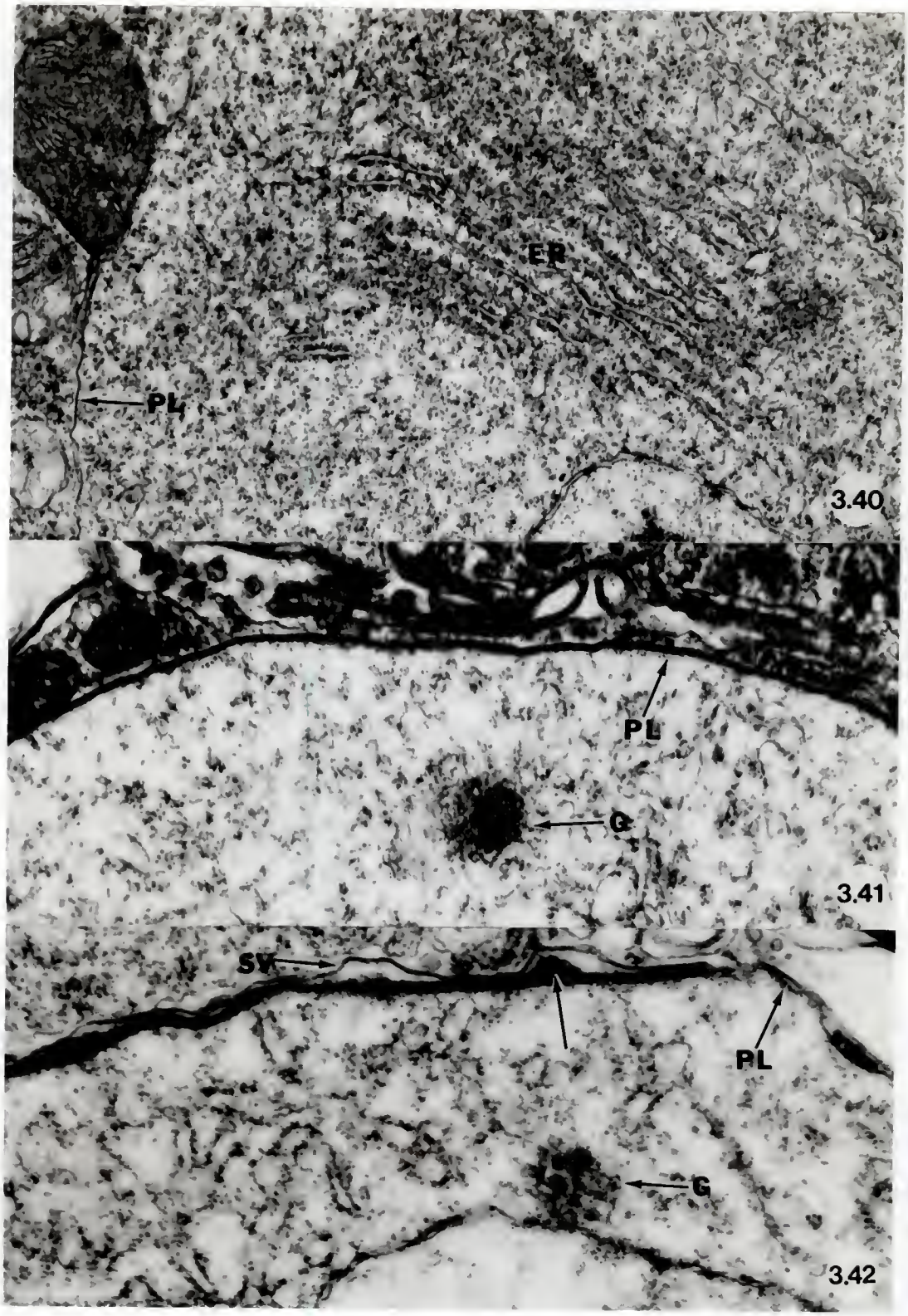


Figures 3.35-3.39. Electron micrographs of selected stages in the sporulation sequence of Amblyospora sp. in larval Culex halifaxi involving nuclear dissociation and ending with pyriform, uninucleate spores. 3.36) Two uninucleate sporoblasts. x10,000; 3.37) Immature spore with vacuolated and compartmentalized polaroplasts. x6800; 3.38) Cross section of the anterior portion of an immature spore. Note that the compartments of the polaroplast are limited by membranous extensions of the polar tube. x20,000; 3.39) Mature uninucleate spore, the product of nuclear dissociation. x8000.

aedis and C. magna, sporogony commonly involved repeated nuclear divisions with delayed cytokinesis. These sporogonial plasmodia divided by multiple fission, producing variable numbers of spores.

Light and ultrastructural evidence on the development and morphology of this Amblyospora sp. has established that it is closely aligned with several established genera. Concurrent sporulation sequences, one involving meiosis, occur in E. aedis (Becnel et al., 1989) and C. lunata (Hazard et al., 1984; Chapter II). In these species, meiosis is abortive and rarely produces normal meiospores but when successful they are similar to the Amblyospora type. In E. aedis, the second sporulation sequence involves nuclear dissociation to produce uninucleate spores like those formed in this Amblyospora species. The second sequence in C. lunata is distinctive by its failure to undergo nuclear dissociation, resulting in binucleate, lanceolate spores. Successful meiosis that ends with normal meiospores is diagnostic for many species of Amblyospora but this report of a second concurrent sporulation sequence is new. In several species, meiospores are infectious to a copepod intermediate host, with sporulation ending with uninucleate spores strikingly similar to those produced in this Amblyospora sp. (Andreadis, 1985a; Sweeney et al., 1985). Culicospora magna also forms a spore that is virtually identical to those in the latter 2 species (Becnel

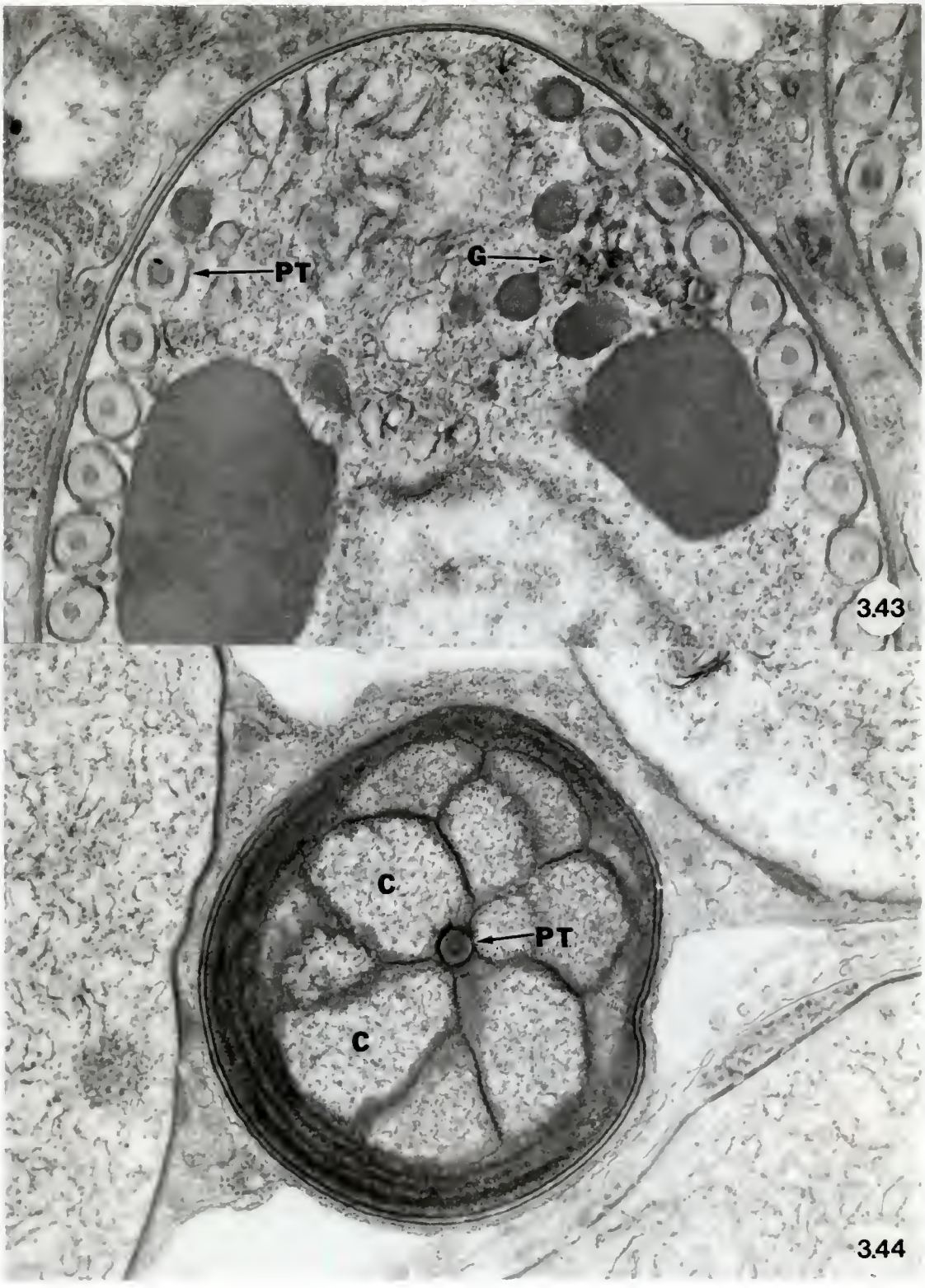
Figures 3.40-3.42. Electron micrographs of selected stages of Amblyospora sp. in larval Culex halifaxi. 3.40) Enlargement of a portion of figure 3.19 showing endoplasmic reticulum (ER) and the plasmalemma (PL). x48,000; 3.41) Enlargement of a portion of figure 3.20 showing the doubled plasmalemma (PL) and Golgi (G). x31,500; 3.42) Enlargement of figure 3.21 showing the sporophorous vesicle (SV), plasmalemma (PL), Golgi (G), and amorphous material (arrow). x42,000.


ER
PL
3.40
PL
G
3.41
SV
PL
G
3.42

et al., 1987). In C. magna this spore is formed as a result of nuclear dissociation in the mosquito host and is directly responsible for horizontal transmission to the mosquito without the involvement of an intermediate host.

Although complete information on the full life cycle is unavailable, the observation of stages in the adult suggests the involvement of one or two horizontal sequences and a third, possibly fourth sporulation sequence (Fig. 3.47). Culex halifaxi is an unusual mosquito that is both predacious and cannibalistic (Debenham, 1988). Cannibalism of infected larvae would provide the opportunity for the uninucleate spores from dissociation to initiate a horizontal sequence in the mosquito. Similar horizontal sequences occur in C. magna and E. aedis involving gametogenesis, plasmogamy and nuclear association (Becnel et al., 1987, 1989). Alternatively, death of infected larvae and ingestion of the meiospores by a copepod intermediate host would initiate a horizontal sequence that ends with spores infectious to the mosquitoes which prey upon the copepods. This pathway would be representative of that known for several species of Amblyospora (Andreadis, 1988a; Sweeney et al., 1988). Regardless of the path, once the parasite enters the mosquito host, a developmental sequence that involves plasmogamy and nuclear association ending with binucleate spores is predicted. These binucleate spores would function to transovarially transmit the pathogen to

Figures 3.43-3.44. Electron micrographs of selected stages of Amblyospora sp. in larval Culex halifaxi. 3.43) Enlargement of a portion of a sporoblast in figure 3.36 showing the involvement of the Golgi (G) in polar tube (PT) formation. x48,000; 3.44) Enlargement of figure 3.38 showing the polar tube (PT) and the chambers (C) of the polaroplast. x42,000.


PT
G
3.43
C
PT
C
3.44

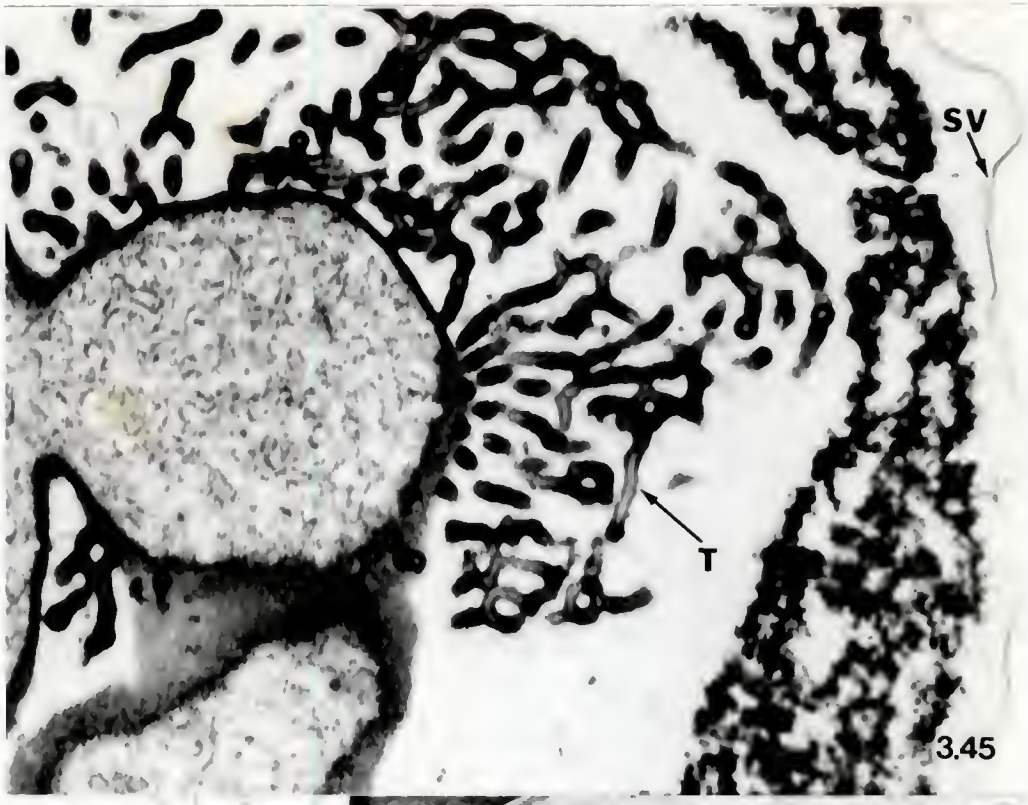
progeny as occurs in other heterosporous microsporidia of mosquitoes.

The present species has affinities for the type species of three established genera based on morphology and development (Amblyospora, Culicosporella, Edhazardia). However, certain aspects of the developmental cycle are unknown, most importantly, the suspected sequence in the adult involving transovarial transmission and the potential role of an intermediate host. For these reasons and until such time as this information is obtained, this present species will be retained within the genus Amblyospora. A species name will be assigned when this work is formally submitted for publication.

Systematics

Amblyospora sp. Becnel, 1986. Characters: heterosporous microsporidium with 2 concurrent sporulation sequences, both arising from diplokaryotic meronts and ending with haploid spores. One sequence dominant, involving meiosis and ending with thick-walled, broadly oval meiospores that measured $4.30 \pm 0.04 \times 3.44 \pm 0.03$ μ (Heidenhain-stain). The other sequence involving nuclear dissociation to produce uninucleate, lanceolate, thin-walled spores that measured $8.47 \pm 0.12 \times 3.72 \pm 0.05$ μ (Heidenhain-stain). Other sporulation sequences are suspected but have not yet been demonstrated. Type host: Culex halifaxi Theobald 1903. Type locality: Queensland, Australia.

Figures 3.45-3.46. Electron micrographs of selected stages of Amblyospora sp. in larval Culex halifaxi. 3.45) Enlargement of a portion of the sporont in figure 3.23 demonstrating the sporophorous vesicle (SV) and tubules (T) attached to the protuberance of the sporont. x23,500; 3.46) Enlargement of figure 3.39 showing the polar tube (PT). x16,000.



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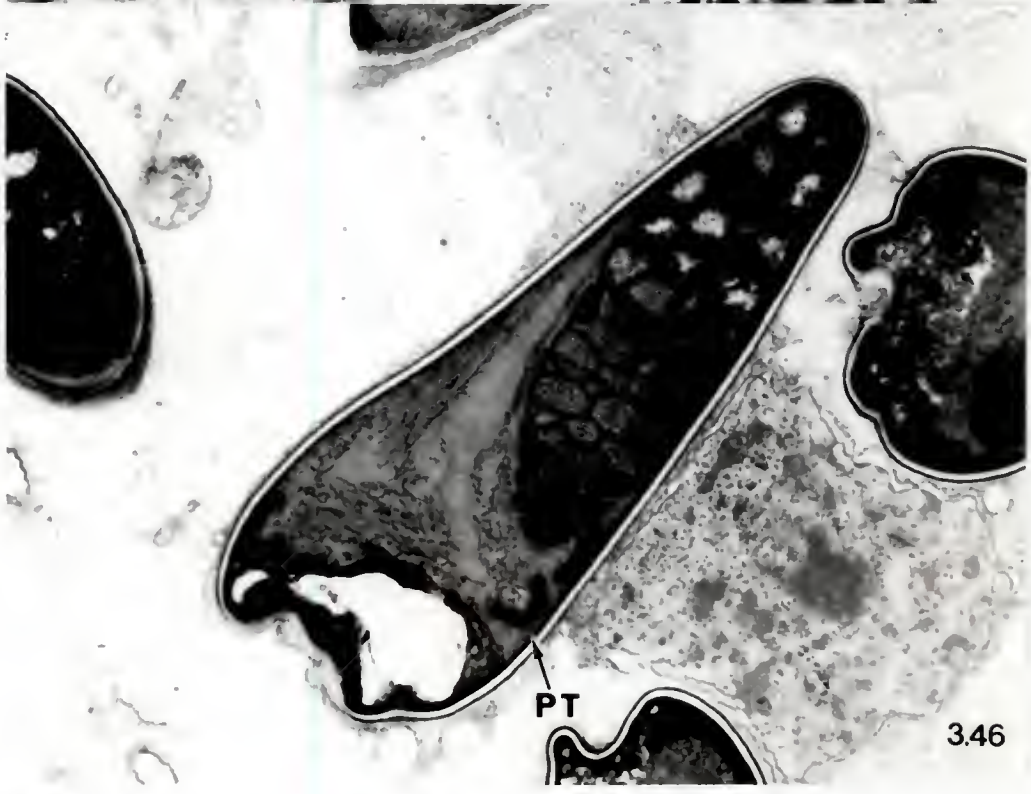

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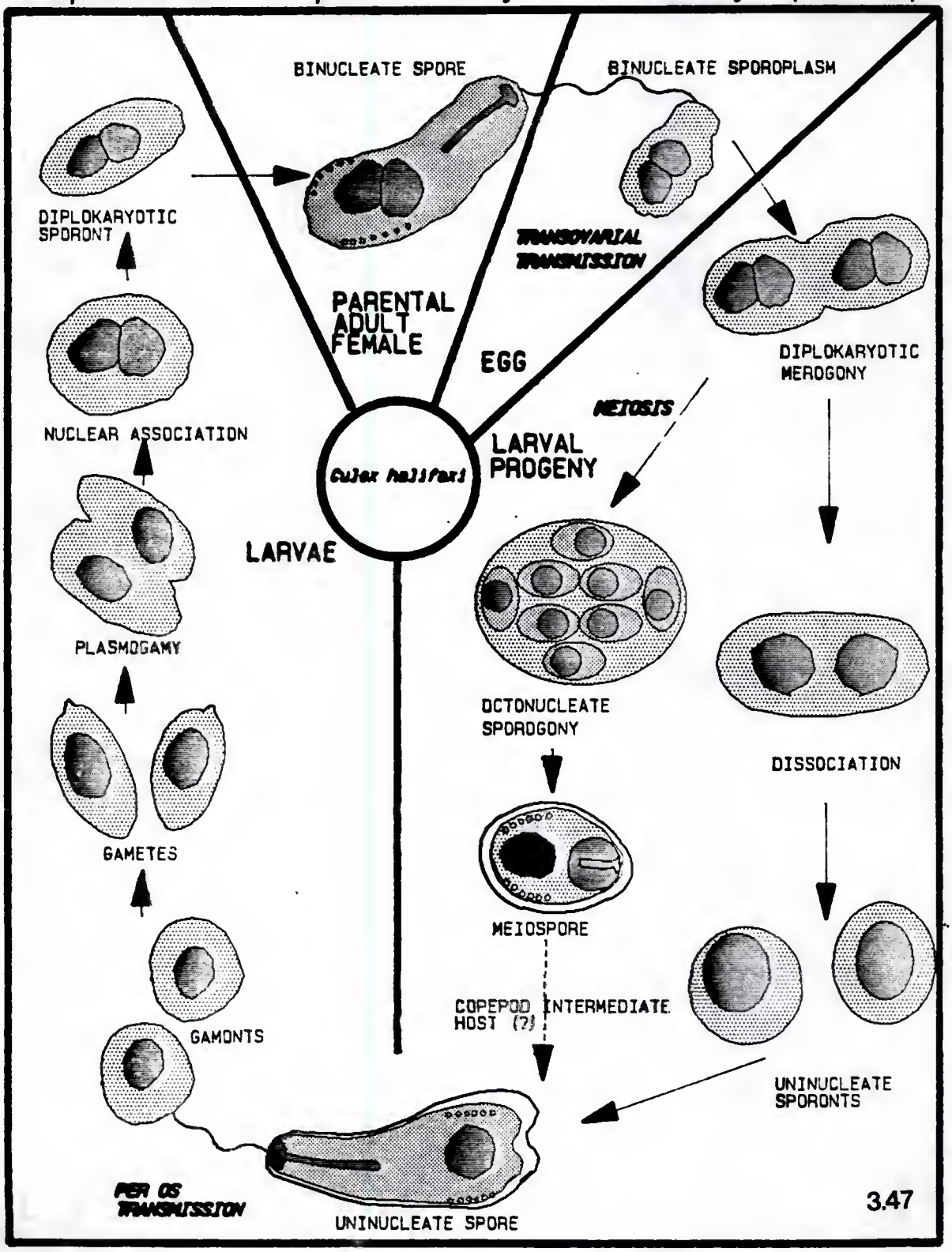
Figure 3.47. Diagramatic representation of the proposed developmental cycle of Amblyospora sp. in the mosquito Culex halifaxi.

Proposed Developmental Cycle of *Amblyospora* sp.



CHAPTER IV
HORIZONTAL TRANSMISSION OF *Amblyospora californica* IN THE MOSQUITO *Culex tarsalis* AND A COPEPOD INTERMEDIATE HOST *Macrocyclops albidus*

Introduction

Amblyospora californica (Kellen and Lipa, 1960) Hazard and Oldacre, 1975 was first described parasitizing the fat body of *Culex tarsalis* larvae and assigned to the genus *Thelohania* Henneguy, 1892 based upon sporonts that typically gave rise to eight spores (Kellen and Lipa, 1960). Subsequently, another microsporidium was found infecting oenocytes of adult *C. tarsalis* females and described as *Nosema lunatum* (Kellen et al., 1967). In a landmark discovery, Hazard and Weiser (1968) reported a new type of dimorphism for the microsporidia characterized by two sporulation sequences (separated in time and host stage) the first giving rise to the second. These researchers provided documentation that in *T. legeri* Hesse and *T. obesa* Kudo, a binucleate spore, of the *Nosema*-type formed in female adult anopheline mosquitoes, was responsible for the infection of progeny. In the infected progeny, a sporulation sequence of the *Thelohania*-type formed spores in groups of eight (meiospores). At that time, it was suggested *T. californica* and *N. lunatum* may represent the same species and later Hazard and Oldacre (1975) combined the two, designating it

as the type species for the genus Amblyospora. Still unanswered was the role of the meiospores in the life cycle as all attempts to horizontally infect larvae failed (Kellen and Wills, 1962a & b; Kellen et al., 1966a & b). Hazard et al. (1979) postulated that the meiospores may function to infect an alternate host prior to reinfecting the primary mosquito host. The breakthrough finally came when Sweeney et al. (1985) discovered that the meiospores of A. dyxenoides Sweeney, Graham and Hazard, 1988 are infectious to a copepod intermediate host.

Several previous reports have presented developmental information on A. californica in C. tarsalis (Kudo and Daniels, 1963; Lipa and Bartkowski, 1981; Chen, 1988). This study was undertaken to: (1) document the two horizontal transmission sequences of development of A. californica (one in the copepod intermediate host, the other in the mosquito); (2) confirm earlier reports for A. dyxenoides (Sweeney et al., 1988) and A. connecticus (Andreadis, 1988); (3) establish new characters for the type species, A. californica. Additionally, for Amblyospora sp. in C. salinarius, the chromosome number is determined and the results of cross infectivity tests has provided new clues relative to the taxonomic identity of this species with respect to A. californica. Finally, the establishment of the developmental cycle of A. californica (the type species for the genus) provides the opportunity to compare and

discuss the relationship of this and other heterosporous microsporidia of mosquitoes.

Materials and Methods

A colony of C. tarsalis Coquillett infected with A. californica (Kellen and Lipa, 1960) Hazard and Oldacre, 1975 and a healthy colony were obtained from Dr. Monica Asman of Berkeley, California. Both colonies originated from the Bakersfield area. The parasite was maintained through transovarial transmission, male progeny dying as a result of the infection. The healthy colony provided both males to maintain the infected colony and larvae for horizontal transmission tests. Culex salinarius Coquillett larvae with meiospores of an Amblyospora (Kellen, Chapman, Clark and Lindegren, 1966) Hazard and Oldacre, 1975 were collected in the Gainesville area for use in cross infectivity transmission studies. Healthy C. salinarius were obtained from colonies maintained at the USDA/ARS Insects Affecting Man and Animals Laboratory, Gainesville, Florida. The copepod Macrocyclops albidus (Jurine) was collected from woodland pools in the environs of Gainesville, Florida.

Horizontal infection of copepods. Copepods were separated into groups of approximately 100 individuals in 250 ml of filtered field water (0.2 μ). These groups were inoculated with meiospores from three fourth instar larvae of either C. salinarius or C. tarsalis (approximately 1 x 10^4 spores / ml) at room temperature. Control groups were

handled in a similar manner but without the addition of meiospores.

Horizontal Infection of Mosquitoes. Spores derived from M. albidus horizontally infected with meiospores as described above, were used as inoculum. Twenty-five 1st or 2nd instar mosquitoes were placed into 60x15 mm Petri dishes containing 20 ml of filtered field water with 3-6 ground or minced infected copepods. A small amount of food was added to each dish and larvae held for 24 hr at room temperature. Larvae were then transferred to laboratory pans that contained 500 ml water and reared according to normal protocol. Control groups were handled in a similar manner but without the addition of spores.

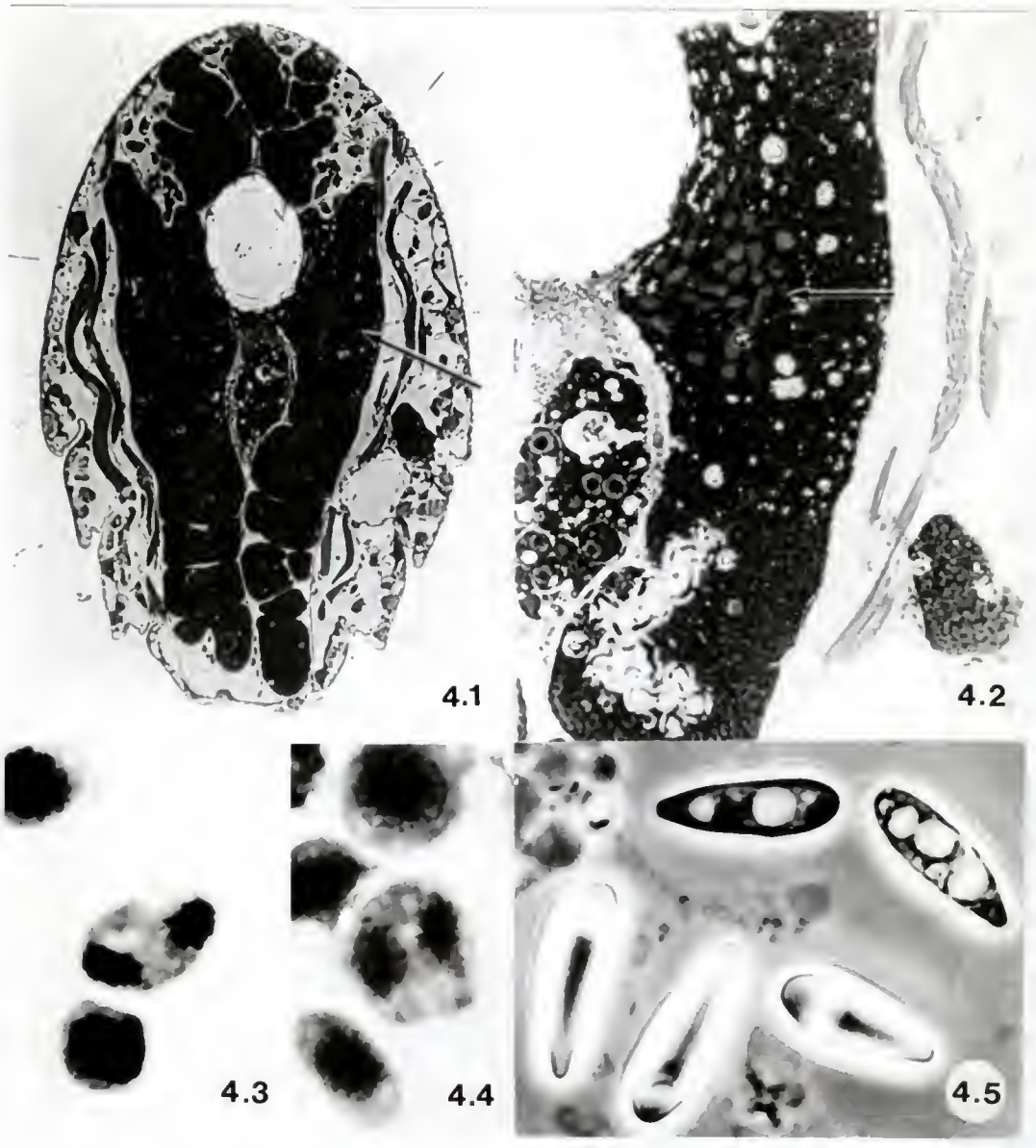
Typically, spores from copepods infected with A. californica were fed to C. tarsalis larvae. In one test, spores from M. albidus infected with Amblyospora sp. from C. salinarius were fed to larvae of both C. salinarius and C. tarsalis.

Development of the pathogen was followed and characterized at the light level with Giemsa-stained smears, Heidenhain-stained paraffin sections and Lacto-Aceto-Orcein (LAO) according to Hazard et al. (1984). Tissues for ultrastructural examination were prepared according to the protocol described in Chapter II.

Results

Two sequential horizontal developmental sequences were required to establish A. californica in a new generation of C. tarsalis. One sequence was in an intermediate host which began with meiospores from mosquito larvae and ended with uninucleate haploid spores in the copepod intermediate host. The other was in larval mosquitoes that began with uninucleate haploid spores from the copepod and ends with binucleate spores in the adult female mosquito (Fig. 4.35).

Development in Macrocyclops albidus. Female copepods exposed to meiospores of A. californica developed ovarian infections (Figs. 4.1, 4.2) which led to the development of uninucleate, haploid spores in 6-14 days. Male copepods were not susceptible. Meronts were round cells with a large nucleus (Figs. 4.3, 4.6) and divided primarily by binary fission (Fig. 4.7) but occasionally quadrinucleate plasmodia were detected. Sporonts initially became elongate (Figs. 4.4, 4.8) and in EM were found to have a doubled plasmalemma, one the primordium of the sporophorous vesicle (Figs. 4.8, 4.30). Sporonts normally divided by binary fission to produce 2 spores but occasionally formed quadrinucleate sporogonial plasmodia that underwent multiple fission in the form of a rosette (Fig. 4.9). Cytokinesis resulted in sporoblasts each within a sporophorous vesicle (Figs. 4.5, 4.10, 4.11). The mature spores were lanceolate, often curved with an extensive polaroplast composed of

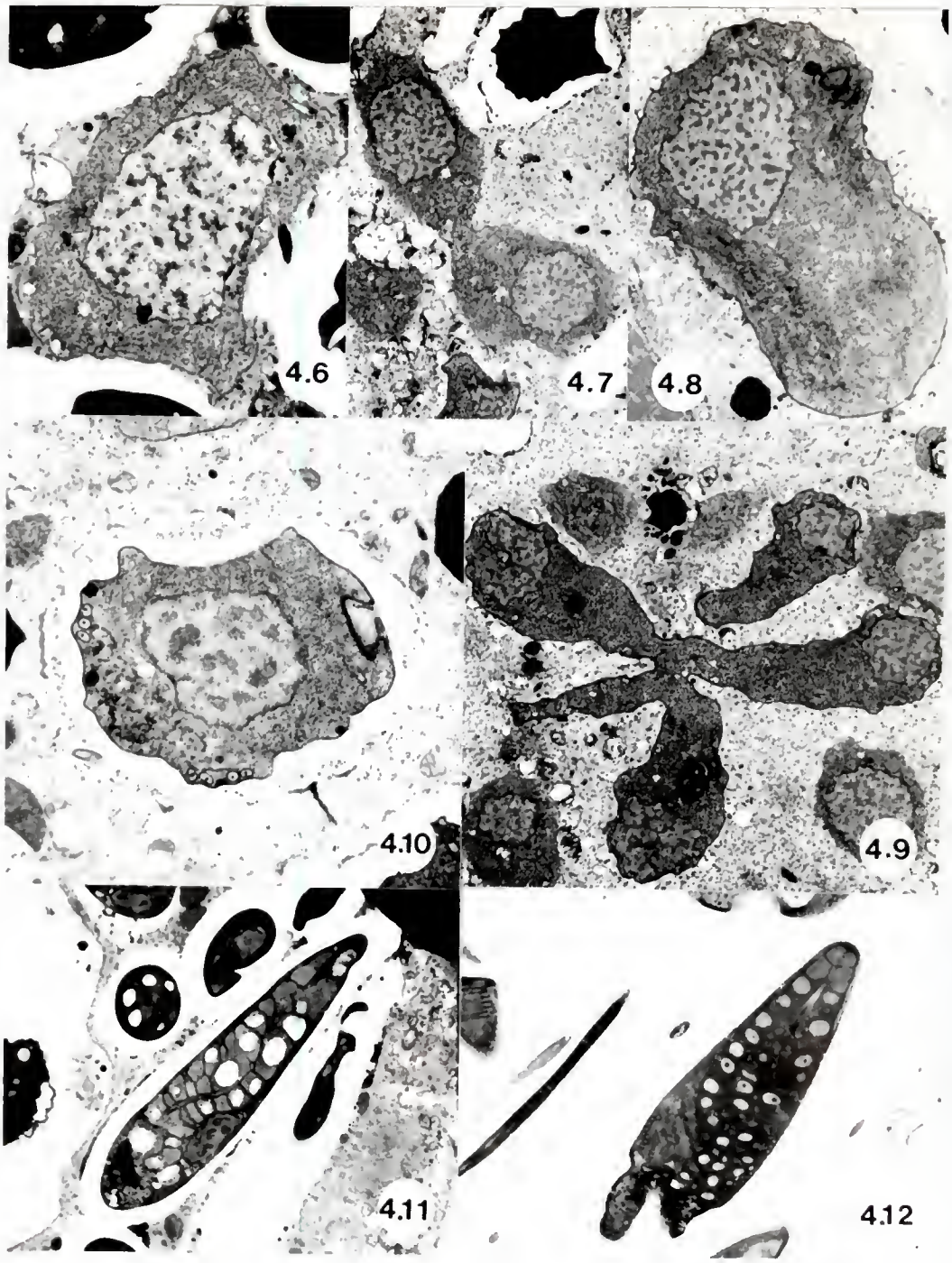


4.1-4.5. Developmental stages of Amblyospora californica in the copepod intermediate host Macrocyclops albidus. 4.1) Histological sagittal section of the copepod with infected ovarian tissues (arrow). x120; 4.2) Vegetative stages in ovarian tissues (arrow). x200; (Figs.4.3-4.5 Giemsa-stained and x2000); 4.3) Meronts and dividing meronts. 4.4) Sporonts and dividing sporonts. 4.5) Sporoblasts and mature uninucleate spores.

numerous compartments and measured 12.50 x 3.84 μ (Figs. 4.5, 4.12). Spores were of the Culicospora-type, each formed within a sporophorous vesicle (Figs. 4.11, 4.12).

Development in Culex tarsalis. Uninucleate spores of A. californica formed in the copepod were infectious to the mosquito (both sexes). Development of the parasite was delayed until late fourth instar larvae and pupae, this pattern is comparable to the transovarial sequence in female progeny of infected females (Becnel 1986). The parasite was found in oenocytes of mosquito pupae and adults (Fig. 4.21), but the path and tissues involved could not conclusively be determined. Some gamonts were detected in "blood" cells, of unknown origin (Figs. 4.13, 4.19), in larvae, where they remained dormant until some factor triggered multiplication. These stages were round in shape with a large nucleus and clumped chromatin (Figs. 4.14, 4.20). Gametes multiplied by binary fission and were characterized by a nipple located at the anterior end of the pyriformed-shaped cell with the nucleus located at the opposite end (Figs. 4.22, 4.23, 4.32). The nipple first appeared as a doubling of the plasmalemma (Fig. 4.33) and developed into a structure resembling a papilla (Fig. 4.34). Plasmogamy (Figs. 4.15, 4.16, 4.24, 4.25) was followed by nuclear association to form the diplokaryon (Fig. 4.26). Merogony was by binary fission (Fig. 4.17) and ended with diplokaryotic sporonts

Figures 4.6-4.12. Electron micrographs of selected stages in the sporulation sequence of Amblyospora californica in the copepod Macrocyclops albidus that ends with pyriform, uninucleate spores. 4.6) Uninucleate meront. x6800; 4.7) Binary fission of meront. x4000; 4.8) Young uninucleate sporont, the primordium of the sporophorous vesicle visible as small blisters. x4000; 4.9) Sporogonial plasmodium dividing in the form of a rosette. x4000; 4.10) Uninucleate sporoblast within a sporophorous vesicle. x6800; 4.11) Immature spore. x4000; 4.12) Mature spore. x5600.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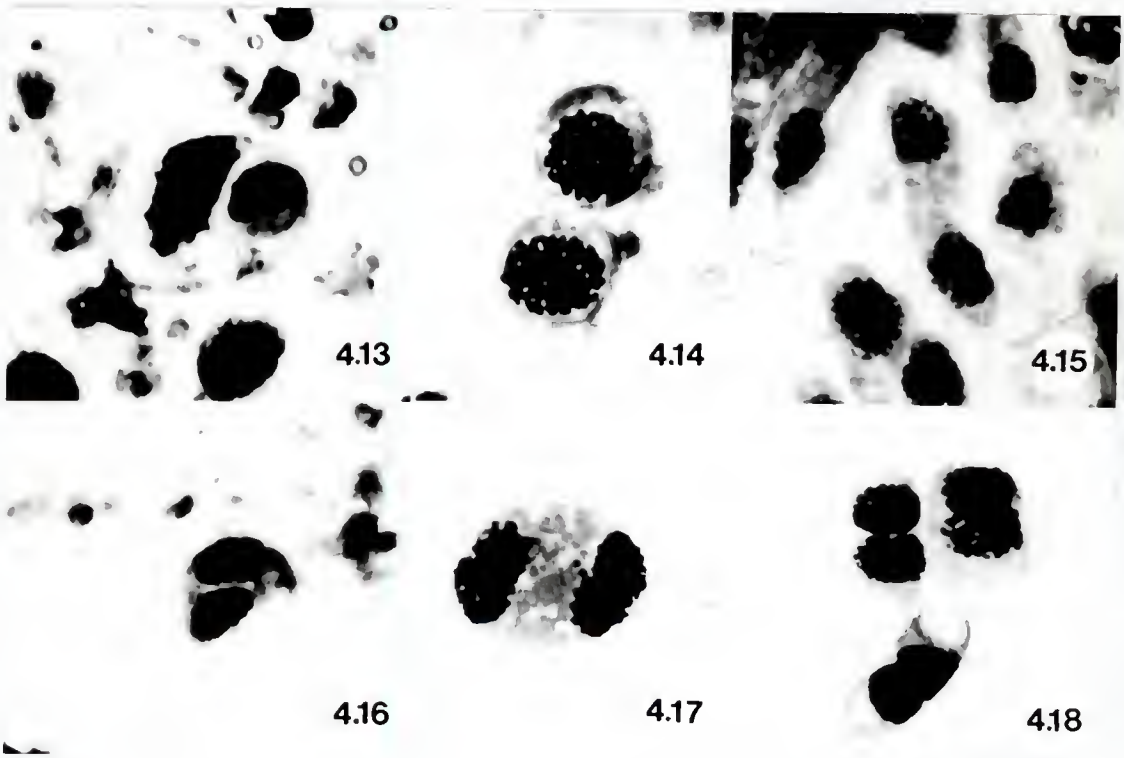
(Fig. 4.18) that transformed into binucleate spores in the adult (Figs. 4.18, 4.27).

Cross infectivity tests. Meiospores of Amblyospora sp. from C. salinarius infected M. albidus and formeduninucleate, haploid spores like those of A. californica. These spores measured 13.23 x 3.85 μ and were infectious to larvae of both C. salinarius and C. tarsalis. A sequence of development, like that described for A. californica, resulted in binucleate spores formed in the adult females of both mosquito species. Male progeny from these infected females developed meiospore infections similar to the Amblyospora sp. in C. salinarius.

Determination of the chromosome number in Amblyospora californica and Amblyospora sp. in Culex salinarius. LAO squashes of both Amblyospora sp. in C. salinarius and A. californica in C. tarsalis during meiosis indicated a haploid number of nine for both species (Figs. 4.28, 4.29).

Discussion

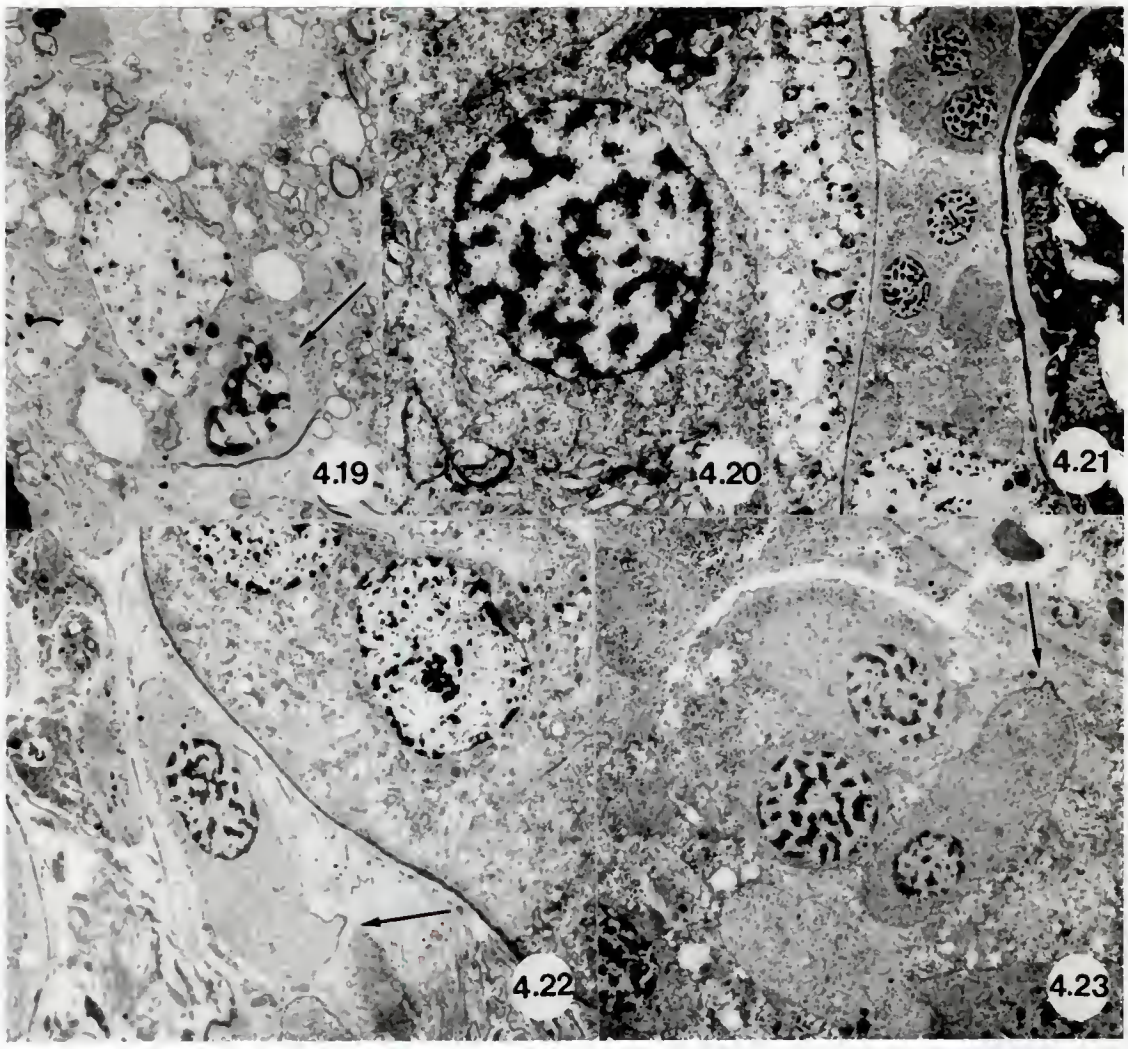
Several reports have confirmed the involvement of an intermediate host in the developmental cycle of the type species A. californica in C. tarsalis but with few details or evidence. Sweeney et al. (1985) reported unpublished information that the life cycle of A. californica had been completed with Mesocyclops leukarti as the intermediate host. Subsequently, Becnel (1986) gave a brief outline of the complete developmental cycle for A. californica



Figures 4.13-4.18. Photomicrographs of the sporulation sequence of Amblyospora californica in Culex tarsalis initiated by uninucleate spores formed in the copepod. Giemsa-stained and x2000. 4.13) Uninucleate stage (gamont) in a host cell. 4.14) Uninucleate meronts. 4.15) Two gametes coming together. 4.16) Plasmogamy of gametes. 4.17) Binary fission of diplokaryotic meronts. 4.18) Binucleate sporoblasts and spore.

reporting the new information that gametogenesis and plasmogamy were involved in the horizontal sequence in the mosquito host. Sweeney et al. (1988) stated that Macrocyclops albidus was also an intermediate host for A. californica, a report that should have been attributed as a personal communication (this was not reported by Becnel 1986). A recent thesis reported the copepod Acanthocyclops vernalis as an alternate host for A. californica but horizontal transmission into the mosquito could not be obtained (Chen, 1988). Based upon the information presented in this study together with previous reports, I can now propose the life cycle for A. californica, compare it with that of two recently described species and discuss the implications of these findings for other species of Amblyospora.

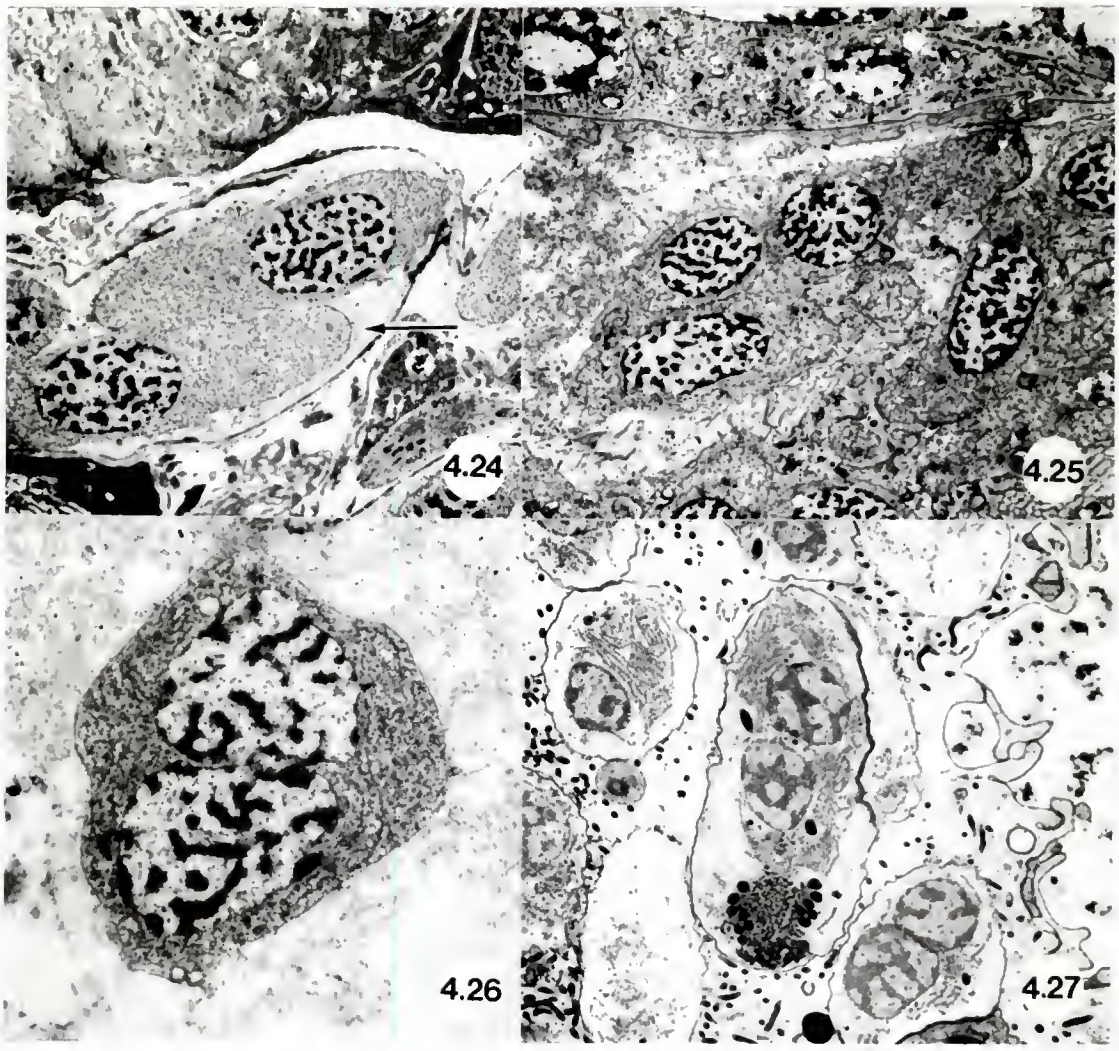
Amblyospora californica is characterized by three sporulation sequences (separated in both time and host), two in the mosquito and one in the copepod intermediate host (Fig. 4.35). One sequence occurs in the fat body of larval male progeny as a result of transovarial transmission and produces meiospores. Death of these larvae releases meiospores, which are infective per os to a copepod intermediate host, into the aquatic environment. The sporulation sequence in ovarian tissues of female copepods ends with uninucleate spores infectious per os to mosquito larvae. In mosquitoes infected per os, a developmental



Figures 4.19-4.23. Electron micrographs of the sporulation sequence of Amblyospora californica in Culex tarsalis initiated by uninucleate spores formed in the copepod. 4.19) Uninucleate stage (gamont) in host cell cytoplasm (arrow). x5000; 4.20) Uninucleate meront. x15,000; 4.21) Gametes in a host oenocyte with a Malpighian tubule to the left and hind gut to the right. x2000; 4.22) Gamete with a papilla at the apex of the cell (arrow). x4000; 4.23) Three gametes, one of which with a papilla at the apex of the cell (arrow). x5000.

sequence that involves gametogenesis, plasmogamy and nuclear association restores the diplokaryotic state. The third sporulation sequence arises from these newly formed diplokaryotic stages to produce binucleate spores in oenocytes of the female adult, an event induced by 20-hydroxyecdysone in Amblyospora sp. from C. salinarius (Lord and Hall, 1983). These spores are infective per ovum to progeny of both sexes. In female progeny, larvae are carriers of the parasite to the adult stage where oenocyte infections repeat the cycle.

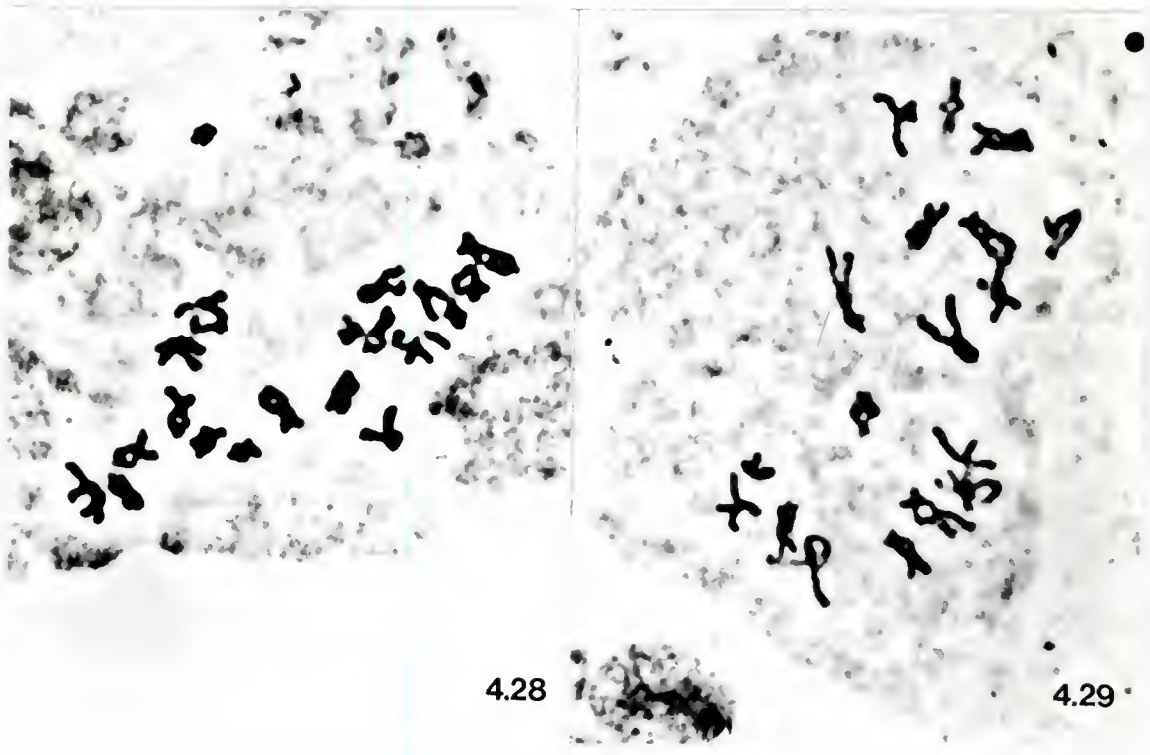
It is noteworthy that in A. californica binucleate spores in females of one generation can give rise directly to binucleate spores in females of the progeny generation via the transovarial transmission cycle. This appears to be the only spore type in the life cycle with this capability. All attempts have failed to reinfect copepods with spores formed in copepods (which would serve to maintain the parasite within the copepod population). These same observations have been made for A. dyxenoides (Sweeney et al., 1988) and A. connecticus (Andreadis, 1988). However, it is reported that in A. culicis meiospores give rise to meiospores in the mosquito host, a significant deviation from other species of Amblyospora (Toguebaye and Marchand, 1986). These studies, however, used field collected larvae and the exact source of the infections were uncertain.



Figures 4.24-4.27. Electron micrographs of the sporulation sequence of Amblyospora californica in Culex tarsalis initiated by uninucleate spores formed in the copepod. 4.24) Two gametes preparing for plasmogamy. Note the thickened plasmalemma at the apex of the lower individual (arrow). x4000; 4.25) Two gametes following plasmogamy, the two nuclei migrating towards one another. x5000; 4.26) Newly formed diplokaryotic meront. x5000; 4.27) Binucleate sporoblast. x6000.

The development and morphology of A. californica as well as the host-parasite relationship are of the same type as reported in A. dyxenoides and A. connecticus with mostly minor differences. However, A. californica has been shown to be less specific with respect to the copepod intermediate host than either A. dyxenoides (Sweeney et al., 1989) or A. connecticus (Andreadis, 1988b). Amblyospora californica has been successfully transmitted to M. albidus and M. leukarti, and the resulting spores were infectious to C. tarsalis to complete the full developmental cycle. Chen (1988) was unable to infect C. tarsalis with spores obtained from Acanthocyclops vernalis cultures. My attempts to infect A. vernalis cultures (the intermediate host of A. connecticus) obtained from Dr. Theodore Andreadis were unsuccessful. The connection between this and the "previous report" isn't clear.

The most significant morphological feature of the gametes in A. californica was the presence of a "nipple-like" structure. This structure has not previously been reported for Amblyospora but is found on the gametes of E. aedis (Hazard et al., 1985; Becnel et al., 1989) and C. magna (Hazard et al., 1985; Becnel et al., 1987). Sweeney et al. (1988) did not study the ultrastructure of the gametes of A. dyxenoides, and Andreadis (1988) was unable to find evidence of this structure on gametes of A. connecticus. Additional ultrastructural investigations on



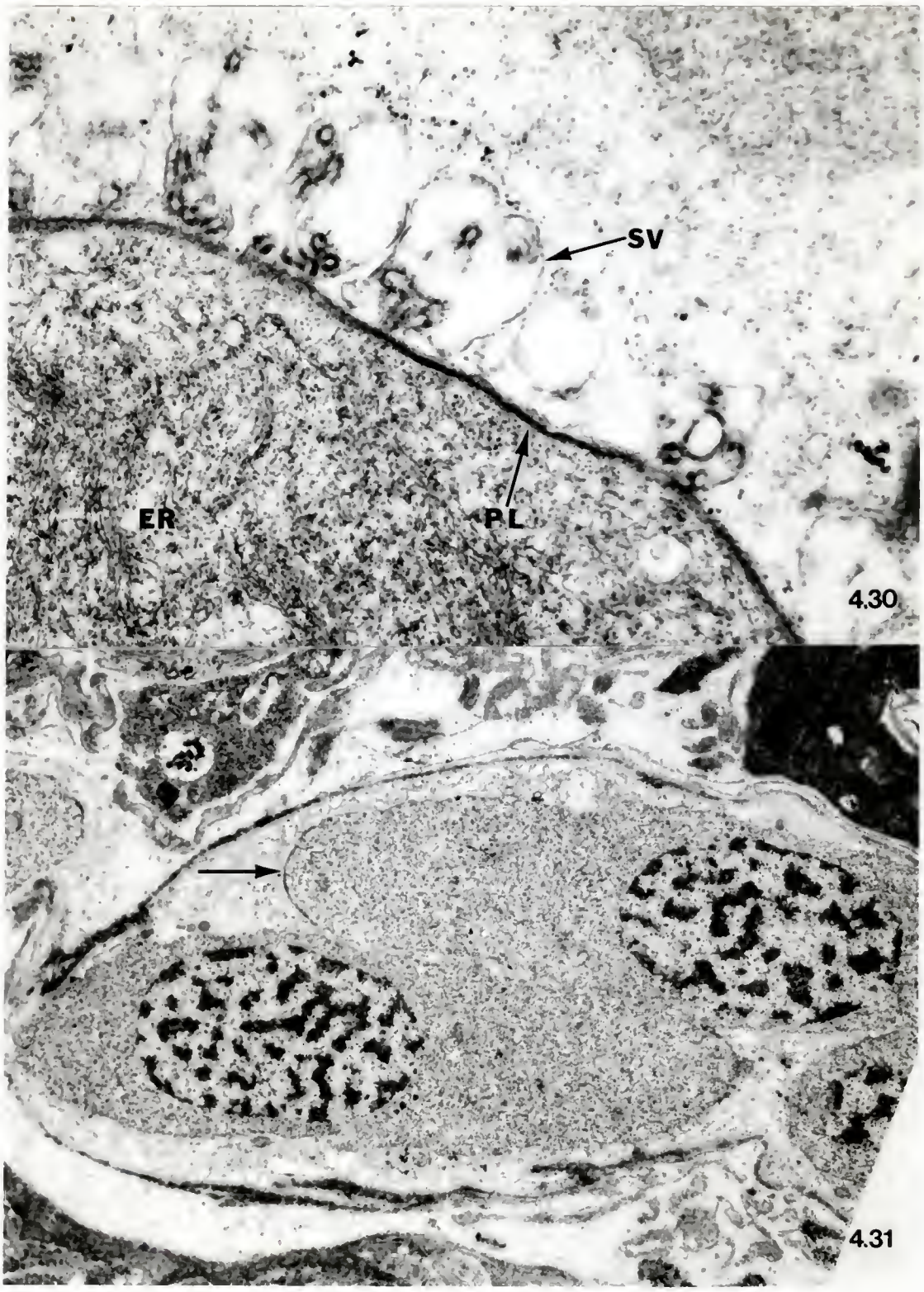
Figures 4.28-4.29. Photomicrographs of meiotic chromosomes as seen in Lacto-Aceto-Orcein stained material. x2500. 4.28) Amblyospora sp. in Culex salinarius indicating nine chromosomes in each of the two nuclei. 4.28) Amblyospora californica in Culex tarsalis indicating nine chromosomes in each of the two nuclei.

the Amblyospora are necessary to determine whether this "nipple" is unique to only certain species or a common feature of gametes in Amblyospora as well as Culicospora and Edhazardia.

The function of the "nipple-like" structure (perhaps better termed a papilla) has not been determined nor is it known to be present on all gametes (ie. isogametes or anisogametes). The papilla often gives the impression of a probing or seeking nature as seen in figure 4.22. The thickened plasmalemma at the apex of one of the two gametes undergoing plasmogamy (Figs. 4.26, 4.31) suggests the presence of a papilla. This structure, found on stages undergoing plasmogamy, suggests a possible role in recognition of the appropriate mating partner and may involve the joining of anisogametes.

The similarities of the morphological features and developmental cycles of A. californica in C. tarsalis and Amblyospora sp. in C. salinarius are the primary reasons that a formal species name has not been assigned to this latter species (Chapman et al., 1966; Andreadis and Hall, 1979a). A recent study comparing these two species concluded that based on morphological, life cycle and polypeptide comparisons, Amblyospora sp. and A. californica are closely related (Jahn et al., 1986). A major reported difference between the two is the haploid number of chromosomes. Hazard et al. (1979) gave the haploid number

Figures 4.30-4.31. Electron micrographs of Amblyospora californica. 4.30) Enlargement of the sporont in figure 4.8 demonstrating the plasmalemma (PL), sporophorous vesicle (SV), and endoplasmic reticulum. x20,500; 4.31) Enlargement of the two gametes in figure 4.24 showing the thickened plasmalemma on the apex of one individual (arrow). x9500.


SV
ER
PL
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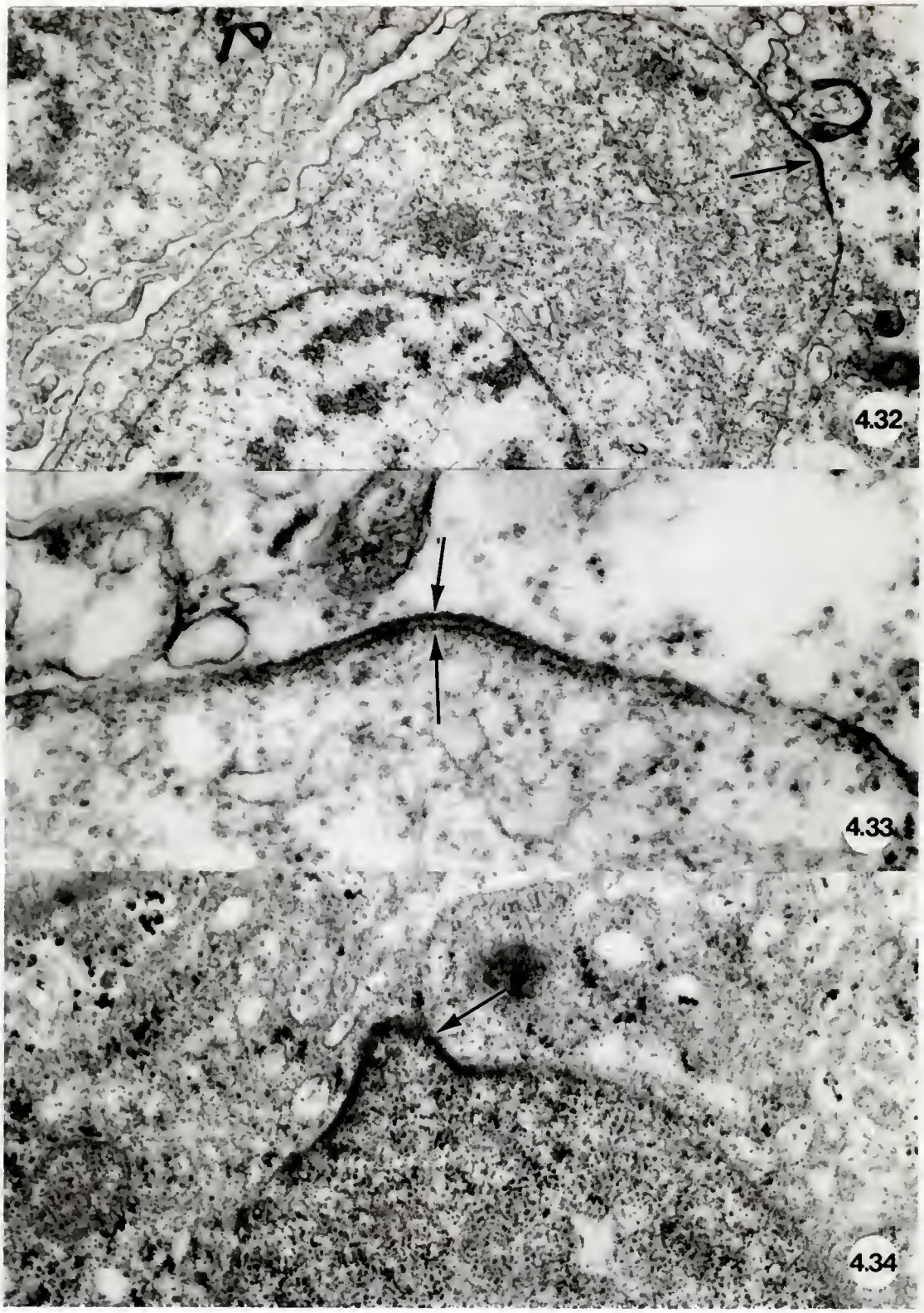
of chromosomes for Amblyospora sp. as seven while Chen (1988) reported that A. californica has a haploid number of nine. My chromosome studies indicate a haploid number of nine for both species. Additional evidence that these two species are closely related are the results of the cross infectivity tests. The same copepod can serve as the intermediate host for both species and the resulting spores are similar in morphology and size. The Amblyospora sp. from C. salinarius can successfully complete its full developmental cycle in both mosquito hosts. This represents the first report of an Amblyospora sp. from one host successfully completing its full developmental cycle in a different mosquito host. Andreadis (1989) successfully transmitted A. connecticus to four alternate mosquito hosts but produced binucleate spores in the adult of only one species. However, A. connecticus was not able to infect the ovaries and complete its developmental cycle via trans-ovarial transmission in any alternate host.

These findings suggest that in addition to ultra-structural and life cycle studies, transmission testing should be conducted prior to the designation of new species of Amblyospora. Until alternative methods of determining the uniqueness of species are established (Streett and Briggs, 1984; Vossbrink and Woese, 1986; Vossbrink et al., 1987), transmission testing may be the only reliable method

Figures 4.32-4.34. Electron micrographs of Amblyospora californica. 4.32) Gamete with the beginnings of a papilla on the apex of the plasmalemma (arrow). x24,000; 4.33) Enlargement of figure 4.32 showing the doubled plasmalemma on the apex of the gamete (arrows). x70,000; 4.34) Enlargement of figure 4.23 demonstrating the papilla (P). x73,500.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to make species determinations of similar Amblyospora spp. in mosquitoes.

Based upon previous reports and the findings of this study, Amblyospora sp. in C. salinarius and A. californica in C. tarsalis are closely related species and in fact may represent only one species. Additional cross transmission tests going from A. californica in C. tarsalis to C. salinarius would provide the additional proof necessary to reevaluate the distinctness of these species.

Relationships of microsporidian parasites of mosquitoes. Conflicting classification schemes usually result from a lack of agreement on the characters that best reflect phylogenetic relationships. This is particularly true of microsporidian taxonomy. Classification of the microsporidia remains, for the most part, in the descriptive stage (Sprague, 1977, 1982; Weiser, 1977; Issi, 1978). Early descriptions were based upon light microscopy observations of sporulation stages with spore shape and size, as well as the host group, usually used to characterize a species (Sprague, 1977). In recent years, ultrastructural investigations of microsporidia have provided important new characters for defining taxa. These ultrastructural characters are primarily of the sporulation stages particularly features of the spores (Issi, 1986, Larsson, 1988). Developmental cycles and host-parasite relationships have not been emphasized in taxonomy largely

due to the paucity of information available for most species. This is understandable for many species where the developmental cycle of the host(s) is extremely long and the parasite has adapted. The finding that an intermediate host is involved in some (probably all) species of Amblyospora complicates matters even further (Sweeney et al., 1985). Certain described species may actually represent only a portion of the life cycle of a heterosporous microsporidium. The impact of these findings on microsporidian taxonomy are yet to be determined. For these and other reasons it would be desirable to accurately predict the complete developmental cycle of a particular species with information on only part of that cycle. Details on both the morphological aspects and developmental cycles of some heterosporous microsporidian parasites of mosquitoes has been presented in this and previous reports. Using this information to understand the relationships of these species may provide clues useful in inferring phylogenies of other groups of microsporidia.

Microsporidia of mosquitoes are particularly well known for a number of reasons. Because mosquitoes are medically important, they have been surveyed worldwide for parasites. Consequently, many of the microsporidian parasites of mosquitoes have been isolated. Conclusions, therefore, can be made as to the more successful forms based on their occurrence. This is not possible for many groups that have

not been so thoroughly investigated. The short life cycles of mosquitoes and colonization of many species has allowed thorough laboratory studies on transmission and developmental cycles. This has greatly facilitated the studies on presporulation stages of these parasites. Finally, there appears to be a sufficient diversity of forms with long standing relationships with their hosts to suggest possible directions of microsporidian evolution.

The most commonly isolated microsporidia from mosquitoes belong to the genus Amblyospora, found in more than 70 host species representing 10 genera worldwide (Hazard, 1977b; Hazard and Oldacre, 1975; Hazard and Chapman, 1977; Castillo, 1980; Daoust, 1981). Next would be members of the genus Parathelohania reported from more than 45 mosquito hosts representing 5 genera worldwide but most commonly from anopheline mosquitoes (Hazard, 1977b; Hazard and Oldacre, 1975; Hazard and Chapman, 1977; Castillo, 1980; Daoust, 1981). These two groups are similar in morphology and development with some species of Parathelohania requiring a copepod intermediate host (S. W. Avery, personal communication). While individual species show some variations, these groups are distinguished by having three successful sporulation sequences in two host species (Fig. 4.35). Binucleate spores formed in the female adult are responsible for transovarial transmission to progeny. A meiotic sequence in some progeny results in meiospores

infectious to a copepod intermediate host. In the copepod host, lanceolate spores (uninucleate by direct descent from the meiospores) are responsible for horizontal transmission to the mosquito host. Other heterosporous microsporidian parasites of mosquitoes are similar in morphology and exhibit variations on this basic developmental theme but they are all monotypic groups. E. aedis has 3 sporulation sequences but completes its development in one host and produces abortive meiospores (Becnel et al., 1989). Amblyospora sp. in C. halifaxi presumably has the same 3 sporulation sequences as E. aedis in the same host but the meiospores are successfully formed and predominant. This suggests a possible fourth sporulation sequence in a copepod intermediate host (Fig. 3.47). Culicosporella lunata also has 3 sporulation sequences in one host and produces abortive meiospores (Fig. 2.43). It is distinguished by its failure to undergo nuclear dissociation and therefore the lanceolate spores are diplokaryotic. Culicospora magna has 2 sporulation sequences in one host but meiosis has never been observed in this species (Becnel et al., 1987). It therefore appears that the most successful groups have the most complex developmental cycles when compared with other heterosporous microsporidian parasites of mosquitoes. Certain characteristics of some of these microsporidian parasites are presented in Table 4.1.

Table 4.1. Characteristics of certain heterosporous microsporidian parasites of mosquitoes.

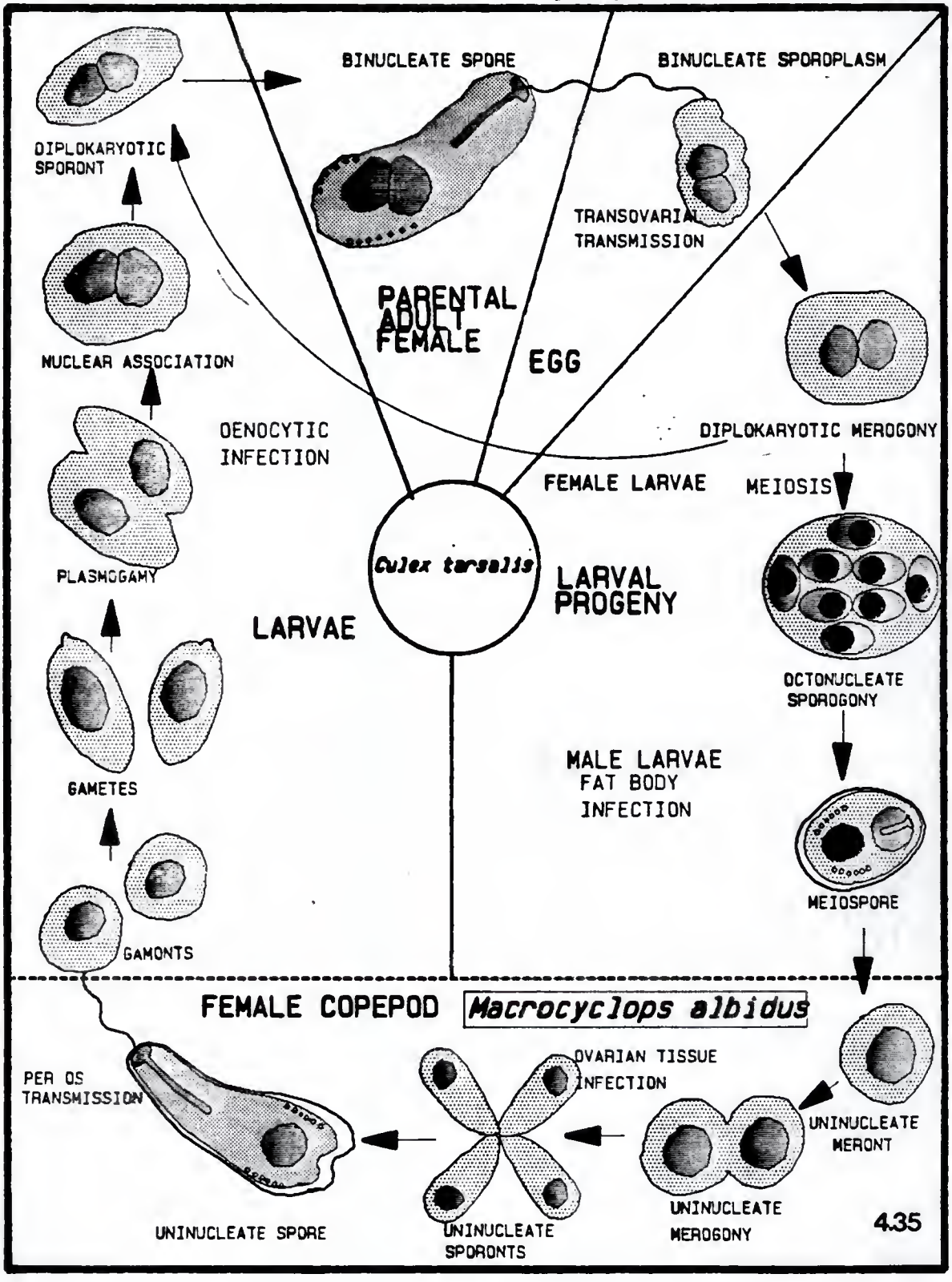
	Mono-typic	Host Specific	Sporulation Sequences	Meiosis	Intermediate Host	Alternate Generation
Australian *Amblyospora* sp.	yes	yes?	4?	yes	yes?	yes
Amblyospora californica	no	yes	3	yes	yes	yes
Parathelohania anophelis	no	yes	3	yes	yes	yes
Edhazardia aedis	yes	no	3 (1 abort)	yes (abort)	no	yes
Culicosporella lunata	yes	yes?	3 (1 abort)	yes (abort)	no	no
Culicospora magna	yes	yes	2	no	no	yes

Specialization is often a consequence of complexity where older parasites (evolutionarily) have become too specialized to produce mutants capable of completing their developmental cycles in a new host (Cameron, 1958). The parasites have therefore lost the ability to adapt to any but closely related hosts. Using this reasoning, E. aedis appears to be a newer parasite (evolutionarily) than Amblyospora or any of the other groups discussed because of its much broader host range.

Meiosis is an integral aspect of the developmental cycle of Amblyospora and Parathelohania with the meiospores only functional in an intermediate host. In E. aedis and C. lunata, meiosis is abortive and apparently not an important aspect of the developmental cycle. C. magna has a developmental cycle functionally like these latter 2 species (vertical transmission followed by horizontal transmission in the one host) but with no evidence suggesting a meiotic sequence. This suggests that the production of meiospores has undergone either progressive or regressive evolution with these species representing an evolutionary series. There is no agreement among biologists as to whether meiosis arose once or many times within the eukaryotes (Penny 1985) but it seems likely that meiosis would develop only once within a specific group. It is compelling to suggest that in some of these monotypic groups, the trend is away from meiosis and simpler developmental cycles that don't involve

Figure 4.35. Diagramatic representation of the developmental cycle of Amblyospora californica in the mosquito Culex tarsalis and the copepod Macrocyclops albidus.

DEVELOPMENTAL CYCLE OF *Amblyospora californica*



an intermediate host. The Australian Amblyospora sp. in C. halifaxi perhaps represents a stable adaptation to a specific environment. Haplosis by nuclear dissociation may represent the first step in this adaptation, bypassing meiosis and the intermediate host. C. magna then may represent the culmination of this transition to a simpler form with no energies expended on meiosis or reliance on an intermediate host.

CHAPTER V
SUMMARY AND DIRECTION OF FUTURE RESEARCH

Heterosporous microsporidian parasites of mosquitoes are a diverse group of organisms with complex life cycles. Recent studies and the information presented in this dissertation on these parasites have provided new information on life cycles in general as well as specific details on morphology and developmental sequences. Aspects of the host-parasite relationships as well as similarities and differences in developmental stages and sequences has clearly demonstrated a close relationship for this unique group of mosquito parasites. This study has provided important new findings on two established microsporidian species and the formal description of a new species from an Australian mosquito.

Culicosporella lunata was characterized by two sporulation sequences each producing morphologically distinctive spores in C. pilosus larvae (Fig. 2.43). Both sequences were presumably derived from a common diplokaryotic cell which in one sequence underwent abortive meiosis to sometimes form anomalous meiospores. In the other sequence, thin-walled diplokaryotic spores were produced from diplokaryotic sporogonial plasmodia. The

ultrastructural characterization of the developmental sequences and spore morphology have clearly demonstrated an affinity to other members of the Amblyosporidae. In addition, a previously unknown sporulation sequence in female adults is revealed that produces binucleate spores. This strongly suggests the involvement of transovarial transmission in the developmental cycle of this species.

A new Amblyospora sp., from the predacious Australian mosquito C. halifaxi, is described based on both light and electron microscopy. This species is a heterosporous microsporidium with two concurrent sporulation sequences, both arising from diplokaryotic meronts and ending with haploid spores (Fig. 3.47). One sequence was dominant and involved meiosis to produce thick-walled, broadly oval meiospores. The other sequence involved nuclear dissociation to produce lanceolate, thin-walled spores. Horizontal transmission to the mosquito host by distinctly different pathways are suspected but have not been demonstrated.

Two sequential horizontal developmental sequences were required to establish A. californica in a new generation of C. tarsalis. Evidence at both the light and ultrastructural levels documents the horizontal sequences of development in the copepod and mosquito hosts. Meiospores of A. californica are infectious to an intermediate copepod host Macrocyclops albidus. The resulting spores are infectious

to larval C. tarsalis giving rise to a sequence that involves gametogenesis, plasmogamy and nuclear association to form binucleate spores in female adults.

Using Lacto-Aceto-Orcein squashes, the chromosome number for an Amblyospora sp. in C. salinarius is shown to be nine, the same as A. californica. Additionally, Amblyospora. sp. from C. salinarius was successfully transmitted to C. tarsalis and completed its full developmental cycle. These findings indicate that this Amblyospora sp. and A. californica are closely related with the strong probability that they represent only one species.

In just the past five years, tremendous advances have been made in solving the complex developmental cycles of heterosporous microsporidia in mosquitoes, some of which involve an intermediate host. The taxonomic implications of these findings for the Microspora as a group are presently being evaluated but the impact will without doubt be far reaching. With the microsporidia in mosquitoes serving as the "template" to compare and make predictions about microsporidia in other groups, future researchers should consider and accept this responsibility and direct their work accordingly. Areas of research to pursue are the following:

1. Conclusive documentation of the complete life cycles of C. lunata in C. pilosus and the Amblyospora sp. in C. halifaxi.

2. Cross transmission testing with A. californica and the Amblyospora sp. in C. salinarius in mosquito hosts as well as copepod hosts. This is necessary to determine the specificity of these parasites and to determine whether they represent one or two species.

3. The genetics of the microsporidia are poorly understood and requires in depth study which should include such basic information as: A) karyotyping; B) interpretations of meiosis; C) karyogamy and its position in the developmental cycle.

4. Ribosomal RNA sequencing together with the detailed information on morphology and life cycles provides the opportunity to determine characters most useful in determining phylogenetic relationships.

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BIOGRAPHICAL SKETCH

James J. Becnel was born in Lake Charles, Louisiana on September 15, 1953. He attended secondary school in Iowa, Louisiana, after which he entered Tulane University in New Orleans, Louisiana, and received a B.S. in biology in 1976.

After a period of time employed as a carpenter, he returned to academia and entered McNeese State University in Lake Charles. During his graduate work, he had a teaching assistantship as well as holding a part-time position at the USDA/ARS Gulf Coast Mosquito Research Laboratory where he began his work on mosquito pathogens. He was awarded his M.S. in zoology in 1981 and became a permanent employee of the USDA/ARS. He was transferred to the Insects Affecting Man and Animals Laboratory in Gainesville, Florida, in 1985 where he is presently employed as a research biologist.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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